REMARKS

Claims 4, 5, 17, 18, 28, and 29 have been amended; these amendments find support in the claims as previously entered and throughout the specification, and particularly by the specification at page 14, line 10; no new matter has been added. As discussed below, the amendments to the claims have been made in response to the rejections under 35 U.S.C § 112, second paragraph, presented for the first time in the Office Action.

Rejection under 35 U.S.C. § 112, Second Paragraph

Claims 4, 17 and 28, and claims 5, 18, and 29, were rejected under 35 U.S.C § 112, second paragraph, on the basis that these claims allegedly lacked definiteness.

A. Claims 4, 17, and 28 were considered indefinite for reciting "... wherein the recombinant polypeptide is a purified polypeptide". The Office Action stated a perceived indefiniteness as to whether the polypeptide referred to in the claim was a "purified recombinant polypeptide" or a "non-recombinant 'purified polypeptide'" (Office Action, page 2). Applicants do not accede to the basis for the rejection: the phrase "recombinant polypeptide" is used in each of claims 4, 17, and 28 and in the parent claim from which each depends (claims 1, 14, and 27, respectively), therefore the polypeptide referred to in claims 4, 17, and 28 is clearly a "recombinant polypeptide" that is also a "purified" polypeptide. However, in order to place the claims in condition for allowance, claims 4, 17, and 28 have been amended to recite "... wherein the recombinant polypeptide *has been purified*" (emphasis added). This amendment is not a narrowing amendment because it does not add any limitations to the claim.

For at least the above reasons, the basis for the rejection has been traversed and, in any case, has been rendered moot; withdrawal of the rejection of claims 4, 17, and 28 under 35 U.S.C § 112, second paragraph, is respectfully requested.

B. Claims 5, 18, and 29 were considered indefinite due to the use of the phrase "produced by cells"; the Office Action considered it to be unclear whether these cells were "'recombinant cell[s]'" or "non-recombinant cells" (Office Action, page 3). Applicants do not accede to the basis for the rejection: recombinant polypeptides are referred to throughout the specification and in the claims as filed as being produced by host cells into which nucleic acids have been introduced (see for example page 25, line 25 through page 32, line 4; and page 34 lines 3-8 of the specification); such host cells are also referred to as 'recombinant host

cells' (page 14, line 10 and claims 6 and 19). Therefore, in claims 5, 18, and 29 it is clear that the cells producing recombinant polypeptides are recombinant host cells, and for this reason the rejection is inapposite. However, in order to place the claims in condition for allowance, claims 5, 18, and 29 have been amended to refer to "recombinant host cells". This amendment makes more explicit limitations that are implicit in the claims, and is not a narrowing amendment.

For at least the above reasons, the basis for the rejection of claims 5, 18, and 29 under 35 U.S.C § 112, second paragraph, has been overcome and also been obviated; withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, First Paragraph (Enablement)

Claims 1-7, 11-20, and 24-29 were rejected under 35 U.S.C § 112, first paragraph for purportedly lacking enablement.

For at least the following reasons, the grounds for the rejection as stated in the Office Action have been overcome.

The foundation of the Office Action's rejection of the claims appears to be an assertion that a particular substrate must be specified in order for one of skill in the art to practice the claimed assay methods of the invention. The Office Action cites no references in support of this assertion, however. Applicants do not accede to this asserted basis for the rejection, because an understanding of the actual practices of those of skill in the protein kinase art indicates that such assays of kinase activity can be readily performed without a "specific" substrate being provided. For example, it has long been known that the activity of kinases can be assayed using endogenous substrate present in the preparation when kinases are partially purified from cells; in other words, in the absence of any added exogenous substrate. An example of this approach is shown in the enclosed Exhibit 1, a reference from 1975 (Kish and Kleinsmith, 1975, "Chromatin Protein Kinases", *Methods Enzymol* 40: 198-208); the authors state "[t]he general experimental approach employed involves ... [an] assay for protein kinase activity in the absence of exogenous substrate" (pp. 198-199), and "[t]his fractionation procedure ... also demonstrates the presence of endogenous substrate within each column fraction" (pp. 203-204). Therefore, in direct contrast to the unsupported assertions of the Office Action, kinase assays can be performed without *any* specific substrate being added.

As an alternative approach in assaying kinase activity, it has long been standard practice by those of skill in the art to assess kinase activity on a panel of protein substrates, or even on mixtures of proteins. An example of this approach is shown in Exhibit 1 at pp. 204-206 and particularly in the table on page 205; fractions from cell extracts that had exhibited kinase activity on endogenous substrates were also tested on protein substrates such as casein, histone, and nonhistone protein, and the table on page 205 shows that it was possible to demonstrate kinase activity in these assays using *any* of the casein, histone, and nonhistone protein substrates. Another example from 1974 is provided as Exhibit 2 (Corbin and Reimann, 1974, "Assay of Cyclic AMP-Dependent Protein Kinases", *Methods Enzymol* 38: 287-290); these authors state at page 287: "Several proteins may be used as substrates in the assay, including muscle phosphorylate kinase and glycogen synthetase, protamine, adipose tissue lipase, casein, specific histones, and histone mixtures." Although the authors of Exhibits 1 and 2 were studying chromatin protein kinases and cAMP-dependent protein kinases, respectively, there is nothing presented in the Office Action to suggest why a panel of different proteins, or a mixture of these proteins, could not be used as substrates by the skilled artisan in the practice of the claimed assay methods. In fact, a variety of proteins and peptides to be used as substrates for kinases have long been commercially available (see Exhibit 3, GIBCO BRL Life Technologies, 1993-1994, "Catalogue and Reference Guide", pp. 16-13 – 16-17), many of which are stated to be recognized by a variety of protein kinases. Thus, it is a standard practice of those in the art to use a set of available proteins and/or peptides as substrates for kinases in assay procedures, and in contrast to the unsupported assertions of the Office Action, *many* of a variety of potential substrates can serve as substrates for a particular kinase.

The Office Action bases the rejection on an assertion that "it is well known in the art that kinases have specific substrates" and that an identification of such substrates would be necessary for the skilled artisan to practice the claimed assay methods. However, although protein kinases phosphorylate their natural substrates within the context of an amino acid "consensus sequence", it does not seem that kinases are restricted to a narrow set of "specific" substrates under assay conditions. Exhibit 4 (Racker, E., 1991, Methods Enzymol 200: 107-111) states at p. 107 that synthetic peptides having amino acid sequences that differ from the "consensus sequences" of natural substrates can serve as kinase substrates: "Synthetic random polymers of amino acids ... serve as excellent substrates for many protein kinases (PK)." In addition, prior to the filing date of the priority application, methods had been developed by those of skill in the art to create large libraries of potential peptide substrates that can be arrayed on a solid support and then contacted with the kinase. Exhibit 5 (Tegge et

al., 1995, Biochemistry 34: 10569-10577) shows the generation of large libraries of peptides - all possible octameric amino acid sequences - and the use of these libraries to determine *a priori* the optimal substrate sequence for a particular kinase. This Exhibit demonstrates that it is within the skill of those in the art to generate large numbers of different peptides to be used as substrates for kinases, and that a kinase can use more than one peptide sequence as a substrate - Figure 1 of Exhibit 5, for example, shows that PKA can phosphorylate many of the possible random octamer library pools represented in the peptide array. Further, Exhibit 5 indicates that the skilled artisan does not need to have any prior information at all about the substrate "specificity" of a kinase in order to develop an assay for the activity of that kinase.

Taken together, these Exhibits show that, in fact, strict sequence specificity is not required for substrates to be phosphorylated by kinases under assay conditions, consistent with many different proteins and peptides being able to act as substrates for a particular kinase. As evidence of the capabilities and established practices of those skilled in the protein kinase art, these Exhibits demonstrate that it was well within the skill of those in the art to practice the claimed assay methods on any of a variety of substrates, such as commercially available proteins and peptides, or to make an almost unlimited number of peptide substrates for use in assays. Claims 1-7, 11-20, and 24-29 therefore do not lack enablement.

For at least the above reasons, the rejection of claims 1-7, 11-20, and 24-29 under 35 U.S.C § 112, first paragraph, has been overcome, and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, First Paragraph (Written Description)

Claims 1-7, 11-20, and 24-29 were rejected under 35 U.S.C § 112, first paragraph, for allegedly not being described in the specification in a sufficient manner to convey to one of skill in the art that the Applicants were in possession of the invention.

The reason for the rejection as stated in the Office Action appears to be that a "genus" of polypeptides is considered by the Office Action to be involved in the claims, and that Applicants are considered not to have provided description of the invention in light of this genus of polypeptides (see pages 6 and 7 of the Office Action). For at least the reasons presented below, the grounds for this rejection have been overcome.

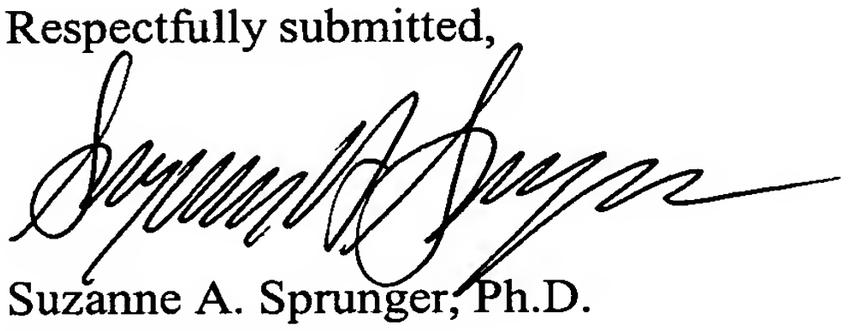
The claims are directed to polypeptides encoded by SEQ ID NO:5 (i.e. SEQ ID NO:11) or SEQ ID NO:13 (i.e. SEQ ID NO:14). The polypeptides of SEQ ID NO:11 and

SEQ ID NO:14 are described in the specification as being related to each other; SEQ ID NO:11 is described at pages 11-12 as a full-length kinase called "QQ3351-BF04", and at pages 12 and 13 of the specification SEQ ID NO:14 is described as a truncated form of QQ3351-BF04. Therefore, the "genus" of polypeptides referred to by the Office Action involves the SEQ ID NO:11 polypeptide and two shorter forms of this polypeptide: Leu-2 through Val-505 of SEQ ID NO:11, and SEQ ID NO:14. The high degree of structural identity among this set of kinase polypeptides does not appear to be consistent with the conclusory statement of the Office Action, that "[t]he claims include species which vary widely in their kinase function" (page 7 of the Office Action). Further, *all* of the amino sequences of these polypeptides - SEQ ID NO:11, Leu-2 through Val-505 of SEQ ID NO:11, and SEQ ID NO:14 – have been completely described in the specification. Applicants were therefore clearly in possession of all of these kinase polypeptides of the invention. In light of the knowledge of those of skill in the art concerning kinase activity assays, as referred to above, the provision of the amino acid sequences of *all* of the kinase polypeptides involved in the claims, and the description in the specification of kinase assays (see, for example, page 38, lines 5-15 and page 42, line 11 through page 43, line 43), Applicants have described the assays of the invention sufficiently for those in the art to practice them, and that is all that is required by 35 U.S.C § 112, first paragraph. A "working example", despite the suggestion of the Office Action (at page 6), is not a requirement for adequate written description when the subject matter of the claims has been sufficiently described by other means.

For at least the above reasons, the grounds for the rejection of claims 1-7, 11-20, and 24-29 under 35 U.S.C. §112, first paragraph, have been overcome, and withdrawal of the rejection is respectfully requested.

If a telephone interview would be helpful in advancing the prosecution of this application, Applicants' attorney invites the Examiner to contact her at the number provided below.

Respectfully submitted,



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that the antibody reacts with the nuclear material. Alternatively, fluorescent-labeled antisera can be used for this purpose.[15]

[15] L. S. Desai, L. Pothier, G. E. Foley, and R. A. Adams, *Exp. Cell Res.* **70**, 468 (1971).

[16] Chromatin Protein Kinases[1]

By VALERIE M. KISH and LEWIS J. KLEINSMITH

The phosphorylation of nonhistone chromatin proteins has been suggested to play a key role in the regulation of gene activity in higher organisms[2-12] (also see Kleinsmith and Kish, this volume [14]). Currently, however, there is little information concerning the enzyme(s) involved in the phosphorylation of these proteins. The nonhistone chromatin phosphoprotein fraction is known to contain an endogenous protein kinase activity which catalyzes the phosphorylation of these proteins in the absence of added exogenous substrate.[3,13] This chapter is concerned with methods for fractionation and assay of these chromatin-associated protein kinases, and will show that chromatin contains a broad spectrum of different types of protein kinases with differing substrate specificities and sensitivity to control by cyclic AMP. The general experimental approach employed involves purification of phosphorylated nonhistone

[1] Studies on this subject in our laboratory have been supported by grants from the National Science Foundation (GB-8123 and GB-23921). V.M.K. held a predoctoral fellowship from U.S. Public Health Service Training Grant 5-T01-GM-72-15.

[2] L. J. Kleinsmith, V. G. Allfrey, and A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.* **55**, 1182 (1966).

[3] T. A. Langan, *in* "Regulation of Nucleic Acid and Protein Biosynthesis" (V. V. Koningsberger and L. Bosch, eds.), p. 233. Elsevier, Amsterdam, 1967.

[4] E. L. Gershey and L. J. Kleinsmith, *Biochim. Biophys. Acta* **194**, 519 (1969).

[5] R. W. Turkington and M. Riddle, *J. Biol. Chem.* **244**, 6040 (1969).

[6] K. Ahmed and H. Ishida, *Mol. Pharmacol.* **7**, 323 (1971).

[7] R. D. Platz, V. M. Kish, and L. J. Kleinsmith, *FEBS Lett.* **12**, 38 (1970).

[8] C. S. Teng, C. T. Teng, and V. G. Allfrey, *J. Biol. Chem.* **246**, 3597 (1971).

[9] M. Kamiyama, B. Dastugue, and J. Kruh, *Biochem. Biophys. Res. Commun.* **44**, 1345 (1971).

[10] N. C. Kostraba and T. Y. Wang, *Biochim. Biophys. Acta* **262**, 169 (1972).

[11] P. B. Kaplowitz, R. D. Platz, and L. J. Kleinsmith, *Biochim. Biophys. Acta* **229**, 739 (1971).

[12] L. J. Kleinsmith and V. G. Allfrey, *Biochim. Biophys. Acta* **175**, 136 (1969).

[13] L. J. Kleinsmith and V. G. Allfrey, *Biochim. Biophys. Acta* **175**, 123 (1969).

EXHIBIT 1

chromatin proteins via salt extraction, chromatography on phosphocellulose columns involving a salt/pH step gradient, and assay for protein kinase activity in the absence of exogenous substrate.

Fractionation Procedure

Preparation of Phosphocellulose Columns

Ten grams of phosphocellulose (Whatman P-11) are suspended in 300 ml of 0.5 *N* NaOH and stirred for 30 minutes at 22°. The fines are removed by suction, and the slurry is then transferred to a small Büchner funnel fitted with a circular disk of Whatman No. 1 filter paper. The phosphocellulose is washed on the funnel with distilled water until the pH of the filtrate is 8 as tested by pH paper. The cake is then transferred to another beaker and stirred with 300 ml of 0.5 *N* HCl for 30 minutes at 22°. The slurry is then washed on the Büchner funnel as described previously until the filtrate approximates a pH of 4. The phosphocellulose is again suspended in 0.5 *N* NaOH and stirred, and is then washed to pH 8 as described above. The resulting cake is suspended in 50 m*M* Tris · HCl, pH 7.5, containing 0.3 *M* NaCl and the pH of the solution is then carefully adjusted to 7.5 using concentrated HCl. The phosphocellulose is stirred at 22° for several hours, and the pH is checked at intervals. Phosphocellulose washed in this manner is then immediately used to pack the column. The excess cellulose is stored at 4° in a tightly closed screw-capped bottle and can be used for packing subsequent columns within the next week. The day before use the phosphocellulose is allowed to come to room temperature and the pH is checked prior to packing.

Freshly washed phosphocellulose gives the cleanest separations of proteins. Storage of washed phosphocellulose at 4° for periods of more than 7–10 days results in protein profiles, which are not as sharply resolved as those resulting from chromatography on freshly washed cellulose. Washed phosphocellulose is slurried in 50 m*M* Tris·HCl, pH 7.5 containing 0.3 *M* NaCl in a ratio of approximately 1:2. A 0.9 × 15 cm column is filled approximately halfway with 50 m*M* Tris · HCl, pH 7.5 containing 0.3 *M* NaCl. After removing bubbles from the bottom section, the bottom of the column which is fitted with a 2-inch section of polyethylene tubing is clamped off and phosphocellulose is pipetted into the column and allowed to settle for 5 minutes. The phosphocellulose should be finely dispersed, with no visible chunks of material present. The bottom is then slowly opened over a period of 10 minutes, and additional slurry is added as the meniscus recedes from the top of the column. When the level of packed phosphocellulose has reached a height approximately

1 cm from the top of the column, the bottom is closed off and the column is replumbed in the cold room. An elution flask, fitted with a capillary tube and containing starting buffer at room temperature is then attached to the column at a differential of 65–70 cm and the column bottom is opened. After approximately 2 hours the differential is lowered to approximately 4 cm and the column is allowed to temperature equilibrate at 4° overnight. The column is washed with 75–100 ml of the starting buffer and the optical density and pH of the eluate are checked prior to protein application. Changes in the permeability of the upper surface of the phosphocellulose usually occur during the overnight wash, as evidenced by the obvious clumping of the cellulose in the top 2–5 mm. To avoid irregular bands caused by uneven permeability of the surface, the top few centimeters of adsorbent are stirred up and removed, leaving a smooth, flat surface. The height of the bed is routinely adjusted to 12–12.2 cm. It should be pointed out that column flow should not be interrupted at any time from the beginning of the buffer wash to the end of fraction collecting.

Column Chromatography of Phosphorylated Nonhistone Proteins

The nonhistone chromatin phosphoprotein fraction, with its associated protein kinase activities, is prepared according to the salt extraction method described elsewhere in this volume (see Kleinsmith and Kish [14]). After concentration at 50 psi of nitrogen in an Amicon ultrafilter equipped with a UM-10 membrane, the proteins are either frozen at —25°,[14] or are immediately dialyzed for 12–15 hours at 4° against 100 volumes of 50 m*M* Tris · HCl, pH 7.5 containing 0.3 *M* NaCl prior to fractionation. The fractionation procedure is a modification of that reported by Takeda *et al.*[15] for fractionation of rat liver nuclear protein kinases. All procedures are carried out at 4° unless otherwise noted.

Approximately 1.5–3 mg of the phosphoprotein fraction (670–980 μg/ml) is applied by pipette directly to the top of the bed after the last of the starting buffer has entered the adsorbent. The entire surface is covered with sample quickly to permit uniform penetration of the sample into the bed. When the last of the sample has entered the cellulose, the surface of the bed and the column wall above it are washed with three 0.5-ml portions of starting buffer, each wash being permitted to sink into the cellulose before the next is applied. After the last buffer wash has entered the cellulose, the column is filled and the elution flask containing

[14] Freezing at this step does not appear to impair protein kinase activity.

[15] M. Takeda, H. Yamamura, and Y. Ohga, *Biochem. Biophys. Res. Commun.* **42**, 103 (1971).

starting buffer is attached. Fractions of 1.0–1.2 ml are collected at 15–20 ml per hour at a constant differential of 24–30 cm until 25–28 ml of starting buffer has passed through the column. The buffer above the bed is then changed to 50 m*M* Tris, pH 8.1 containing 0.6 *M* NaCl and elution is continued for another 34–36 ml. At a total volume of 64–66 ml the last step in the gradient is initiated using 50 m*M* Tris·HCl, pH 8.1 containing 1.0 *M* NaCl and an additional 30–35 ml is collected. The protein content of each fraction is measured by ultraviolet absorbance at 280 nm prior to enzyme assay. Since the protein kinases are extremely labile after fractionation (storage at —70° or overnight at 2° results in a significant loss of activity) the enzyme assay is carried out immediately.

Assay of Protein Kinase Activity

Protein kinase activity in each fraction is measured in the presence of endogenous substrate immediately after the protein profile is read. The reaction mixture of 0.3 ml contains 13 μmoles of Tris·HCl, pH 7.5; 0.1–2 nmoles of [γ-^{32}P]ATP (770–3650 mCi/mmole); 7.5 μmoles of magnesium acetate, and 0.2 ml of each column fraction. When 3′,5′-cyclic adenosine monophosphate (cAMP) is added to the reaction (0.3 nmoles) it is first preincubated for 2 minutes at 30° in the presence of protein kinase and buffer before the remaining components of the reaction mixture are added. The reaction is initiated by the addition of magnesium and after 10 minutes in a 30° shaking water bath, 3.0 ml of cold 1 m*M* ATP are added followed by 3.3 ml of cold 10% trichloroacetic acid (TCA) containing 3% sodium pyrophosphate. The diluted reaction mixture is then filtered under vacuum through nitrocellulose membrane filters (Schleicher and Schuell, B-6, 0.45 μm, 25 mm diameter) which are presoaked in 1 m*M* ATP for at least 30 minutes at room temperature. Each filter is washed twice with 5 ml of 5% TCA containing 1.5% sodium pyrophosphate, oven dried, and counted in 5 ml of toluene scintillation fluid.

The final assay conditions were derived on the basis of results of several preliminary experiments. A comparison of nitrocellulose membrane filters with glass fiber filters (Reeve Angel 934-H and 934-AH) in the presence and in the absence of bovine serum albumin as carrier protein demonstrates lower background counts using the nitrocellulose filter; therefore we have routinely used this type of filter in our assay. In order to minimize still further the background radioactivity on nitrocellulose filters, a variety of conditions were employed, with the results summarized as follows: (1) Unlabeled ATP is the most effective agent in which to soak the filters; neither potassium phosphate buffer (20 m*M*)

nor sodium pyrophosphate (3%) results in any significant diminution of background counts, thus suggesting that the ring structure of the ATP may be important in this regard. (2) Terminating the reaction with 5% TCA in the presence or in the absence of 3% sodium pyrophosphate, followed by unlabeled ATP leads to a high background, whereas adding the ATP first, followed by TCA (our standard procedure) results in low background radioactivity. The fact that the order of addition is critical, coupled with the observation that TCA if used alone results in high background levels, suggests that there may be some interaction between the acid and the nitrocellulose filter causing the increased nonspecific retention of labeled ATP. (3) Use of 2 *M* NaCl to stop the reaction (rather than TCA) does not decrease the high background.

It should be pointed out that other methods which monitor the degree of incorporation of ^{32}P into proteins have been reported. Comparison of the nitrocellulose filter procedure with a modification of that described by Reimann *et al.*[16] involving filter paper washed in cold 10% TCA + 1% sodium pyrophosphate followed by propanol:ether substantiates the superiority of the former procedure. Another method which has been reported[17] involves the precipitation of acid-insoluble ^{32}P-labeled material with 10% TCA followed by dissolution of the centrifuged pellet in 0.1 ml of 1 *N* NaOH and reprecipitation by 5% TCA. This precipitate is then collected on glass fiber filters. However, since the phosphodiester bond is alkali labile, it is unclear how this method can be used to monitor ^{32}P incorporation, since much of the ^{32}P will remain in the supernatant after the second acid precipitation. In conjunction with these studies we discovered that the presence of bovine serum albumin (200 μg added after the TCA precipitation step), although not altering the background level of radioactivity, resulted in a significant decrease in the amount of radioactivity retained after filtration of the samples containing protein kinase. Since attempts to replace the albumin with a phosvitin carrier resulted in elevated backgrounds, we examined the retention of ^{32}P in the absence of carrier protein. Under these conditions the observed retention of counts in experimental samples is significantly increased, hence carrier protein is routinely left out of the procedure.

Acrylamide Gel Electrophoresis

The nonhistone chromatin phosphoprotein fractions are phosphorylated with [γ-^{32}P]ATP using the same assay conditions as described above. The reaction is stopped by adding solid urea to a final concentration of 4.0 *M*, followed by dialysis at 22° overnight against 200 volumes of 10 *M* sodium phosphate, pH 7.0 containing 0.1% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate (SDS). Electrophoresis is performed in a 10% SDS-acrylamide gel as described by Weber and Osborn.[18] Gels are sliced at 1-mm intervals, dehydrated at 68° for 1 hour, and counted in 5 ml of toluene scintillation fluid.

In order to assay protein kinase activity in the gels, a modification of the procedure reported by Rubin *et al.*[19] is employed. Gels containing 7.5% acrylamide are prepared from a solution containing 22.2 g of acrylamide and 0.6 g of *N*,*N*′-methylenebisacrylamide, which are dissolved in water to 100 ml and then filtered. The gels are prepared by deaerating 10 ml of 0.26 *M* Tris·HCl, pH 8.1 followed by addition of 6.75 ml of the acrylamide solution and 2.25 ml of water. After additional deaeration, 0.025 ml of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 1.0 ml of a freshly made 1% solution of ammonium persulfate are added. The gels are polymerized in 6 mm (i.d.) × 75 mm glass tubes after layering 3–4 mm of water above the gel surface. Protein kinase samples are dialyzed overnight at 4° against 200 volumes of 0.13 *M* Tris·HCl, pH 8.1 prior to use. Before protein is added, the polymerized gels are prerun for 30–60 minutes at 2.5 mA/gel using 0.13 *M* Tris·HCl, pH 8.1 in both the upper and lower buffer chambers. Dialyzed samples of 50–100 μl are mixed with an equal volume of 40% sucrose containing bromophenol blue marker and are then layered on top of the gel column. Electrophoresis is carried out at 2.5 mA/gel until the marker reaches the bottom of the gels (approximately 8 hours). Gels are then sliced at 1-mm intervals, and each slice is eluted overnight at 4° in 250 μl of 50 m*M* Tris·HCl, pH 7.5 containing 4 m*M* 2-mercaptoethanol. Aliquots of the eluate are then withdrawn and assayed for protein kinase activity as described previously.

Typical Results[20]

When the purified nonhistone phosphoproteins are chromatographed on phosphocellulose columns and the fractions are monitored for protein kinase activity as described above, a complex profile of heterogeneity is seen which routinely consists of at least 12 distinct regions of enzyme activity (Fig. 1). This fractionation procedure thus emphasizes the multiplicity of the protein kinases associated with the nonhistone chromatin

[16] E. M. Reimann, D. A. Walsh, and E. G. Krebs, *J. Biol. Chem.* **246**, 1986 (1971).
[17] J. Erlichman, A. H. Hirsch, and O. M. Rosen, *Proc. Nat. Acad. Sci. U.S.* **68**, 731 (1971).
[18] K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406 (1969).
[19] C. S. Rubin, J. Erlichman, and O. M. Rosen, *J. Biol. Chem.* **247**, 36 (1972).
[20] For a detailed description of results see V. M. Kish and L. J. Kleinsmith, *J. Biol. Chem.* **249**, 750 (1974).

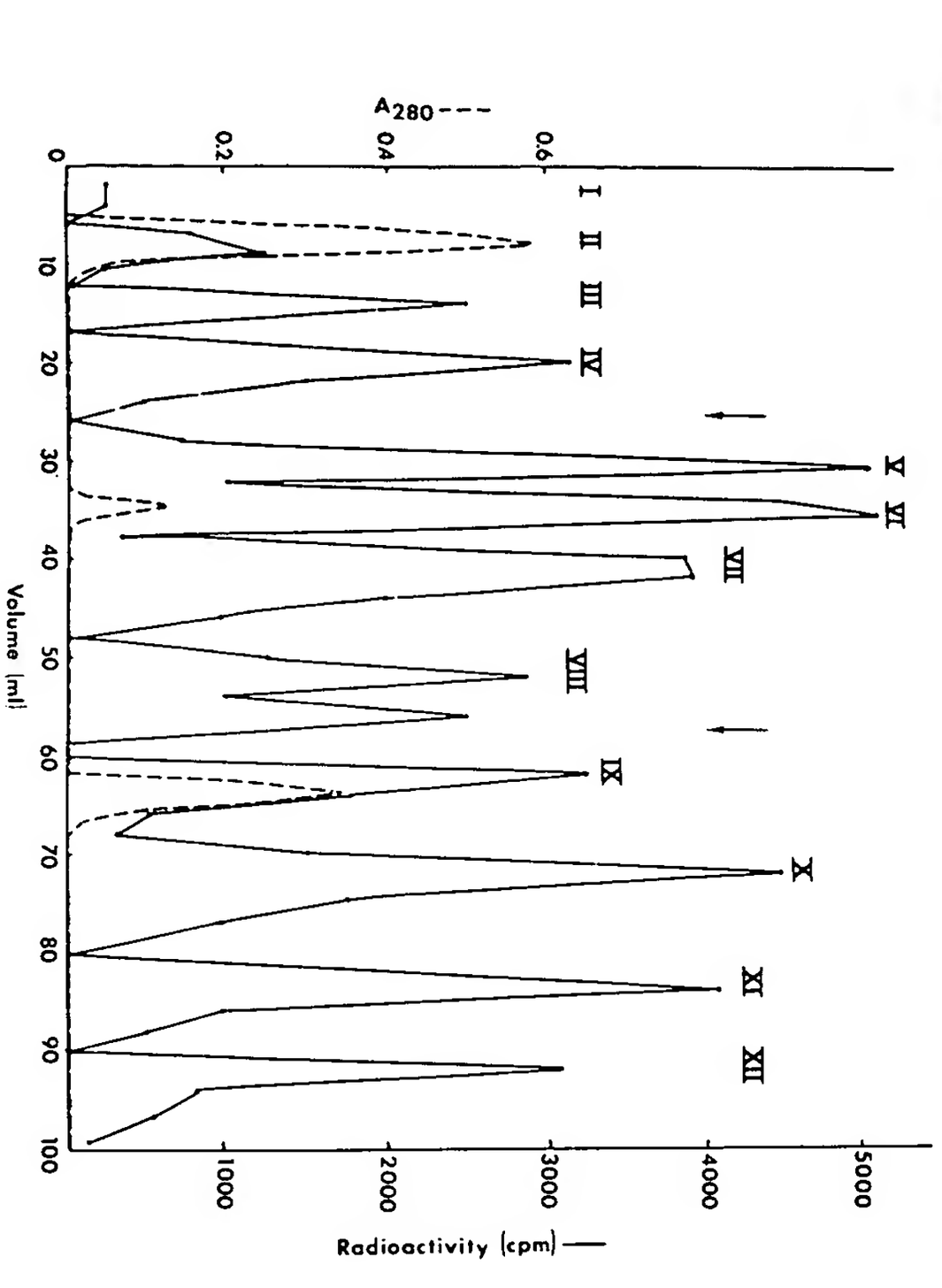

FIG. 1. Phosphocellulose column chromatography of protein kinases associated with the nonhistone chromatin phosphoprotein fraction of beef liver. Approximately 2.5 mg of the protein fraction is applied to the column as described in the text. Arrows mark the salt/pH steps in the gradient. Each column fraction is assayed for protein content by ultraviolet absorption at 280 nm (- - -) and for protein kinase activity by monitoring incorporation of radioactivity from [γ-^{32}P]ATP into protein (●——●) as described in the text. Twelve distinct regions of protein kinase activity are reproducibly distributed throughout the profile, demonstrating the heterogeneous nature of these nuclear enzymes.

phosphoprotein fraction, and also demonstrates the presence of endogenous substrate within each column fraction.

In order to investigate the characteristics of these protein kinases in terms of substrate specificities and response to cyclic nucleotides, it is most convenient to pool various regions eluting from the column. These pools are found to differ from each other in terms of their abilities to catalyze the phosphorylation of different protein substrates. For example, the table shows the results obtained when 4 of the different kinase pools are compared in terms of their abilities to catalyze the phosphorylation of casein, histone, or nonhistone proteins. These data are typical of the rest of the enzyme pools as well, in that each pool has a unique pattern

SUBSTRATE SPECIFICITIES AND EFFECTS OF CYCLIC AMP ON SELECTED NUCLEAR PROTEIN KINASE FRACTIONS[a]

Treatment	Protein kinase activity							
	Fraction I		Fraction VI		Fraction VIII		Fraction XI	
	cpm	% Control	cpm	% Control	cpm	% Control	cpm	% Control
Control	546	100	1029	100	647	100	2113	100
+ cAMP	2883	517	479	47	1910	295	545	26
+ Casein	5	1	1770	172	1039	160	20	1
+ Casein + cAMP	5	1	25	2	3039	469	1751	83
+ Histone	161	30	46	4	6	1	222	11
+ Histone + cAMP	206	38	1800	175	2911	450	530	25
+ Nonhistone protein (NHP)	525	96	1280	124	3950	610	566	27
+ NHP + cAMP	4010	735	1440	140	3882	600	580	27

[a] Fractions I, VI, VIII, and XI from the phosphocellulose column (see Fig. 1) were each pooled and immediately concentrated to a volume of approximately 1 ml by ultrafiltration using a PSAC Pellicon membrane (Millipore). Each pooled fraction was then desalted by passage through a 0.8 × 12 cm column of Bio-Gel P-4 equilibrated with 50 m*M* Tris · HCl, pH 7.5. Under these conditions the protein kinase activity is eluted in the exclusion volume. The above procedures were all carried out at 4°. Protein kinase activity was measured for each pooled fraction in the absence of added substrate as described in the text. This was considered the "control" system. The effects of adding 100 μg of bovine casein (Hammarsten), 180 μg of total calf thymus histone (Type IIA, Sigma), 9–24 μg of a purified nonhistone chromatin phosphoprotein (NHP) fraction (pool II, heated at 60° for 3 minutes to destroy endogenous protein kinase activity), and 1μ*M* 3′,5′-cyclic AMP (cAMP) were also tested in this system. Note that the different kinase fractions have differing substrate specificities and responses to cAMP.

of substrate specificity. These results suggest that each of the pools represents a distinctly different protein kinase activity.

This conclusion is further substantiated when one employs different kinase pools as sources of enzyme for the phosphorylation of nonhistone proteins. This nonhistone protein fraction is known to be highly heterogeneous, containing at least several dozen phosphorylated components which are separable by SDS-acrylamide gel electrophoresis.[7] Thus the obvious question to be asked is whether these protein kinases which have been separated are specific for different components within this nonhistone fraction. When different kinase pools are used to catalyze the phosphorylation of nonhistone proteins and the pattern of labeling is examined by SDS-acrylamide electrophoresis, it is seen that different labeling patterns are obtained (Fig. 2). These results lend further support to our conclusion that the different protein kinase pools actually represent different enzymes.

The effects of the addition of cyclic AMP on the activities of these protein kinases are also uniquely different for each kinase pool. As can be seen in the table, the effects of cyclic AMP in this system are quite complex. Some of the phosphorylation reactions are stimulated, while others are inhibited. The existence of an inhibitory or stimulatory effect is not dependent on the source of the kinase alone, since the same kinase pool may be either activated or inhibited by the cyclic nucleotide, depending on the substrate being phosphorylated. These results again point to the unique characteristics of the different protein kinase pools.

Although we have been routinely dealing with 12 kinase pools for the sake of experimental simplicity, this does not necessarily imply that each of these 12 fractions actually represents a single species of protein kinase. In fact, we have some evidence which suggests that these individual pools may contain more than one enzyme within them. If, for example, pool V is collected and electrophoresed in acrylamide gel under conditions where protein kinase activity is not destroyed, enzyme activity is found in at least two distinct regions of the gel (Fig. 3).

The overall picture which emerges, then, is an extraordinarily large degree of heterogeneity and complexity within the nuclear protein kinases. In order to observe this heterogeneity, however, it is necessary to closely follow the procedures outlined here, since other investigators using somewhat different techniques have not seen this level of complexity.[15,21] Aside from the obvious differences in tissue source and methods of enzyme assay, factors which in themselves may be very important considerations, the most fundamental difference between our work and

[21] R. W. Ruddon and S. L. Anderson, *Biochem. Biophys. Res. Commun.* **46**, 1499 (1972).

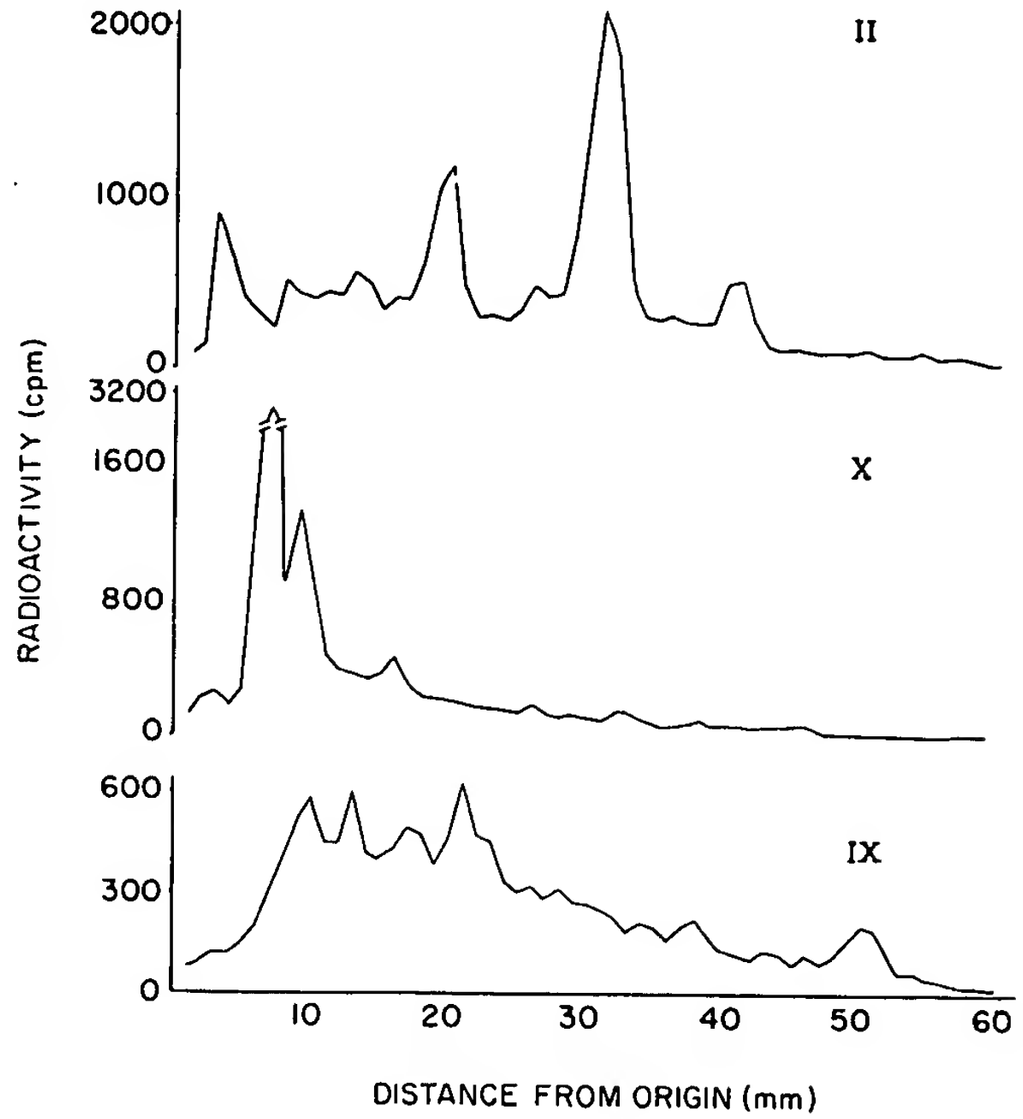

FIG. 2. Phosphorylation of nonhistone chromatin phosphoproteins by different protein kinase fractions. Protein kinase pools II, IX, and X were collected and concentrated as described in the table. The total nonhistone chromatin phosphoprotein fraction used as substrate was preheated at 60° for 3 minutes to destroy its endogenous protein kinase activity. The incubation conditions were as described in the text, except that $MgCl_2$ was used and the incubation was carried out at 37°. After labeling, the proteins were dialyzed and then applied to 10% SDS-acrylamide gels. Note that the distribution of radioactivity in the gels shows that the different kinase fractions are phosphorylating different components of the nonhistone phosphoprotein fraction.

that of others involves the purity of the starting material used for phosphocellulose chromatography. The highly purified nonhistone phosphoprotein fraction which we start with has a very high protein kinase specific activity, thus allowing us to resolve enzyme activities which are not seen when starting with cruder material.

In addition, we have included an additional step in our column elution which increases the complexity of the protein kinase profile by a substantial amount. These enzyme activities are presumably left on the column under conditions employed by others.[15,21] Another factor which may also be involved is the extreme lability of these kinases, which might allow

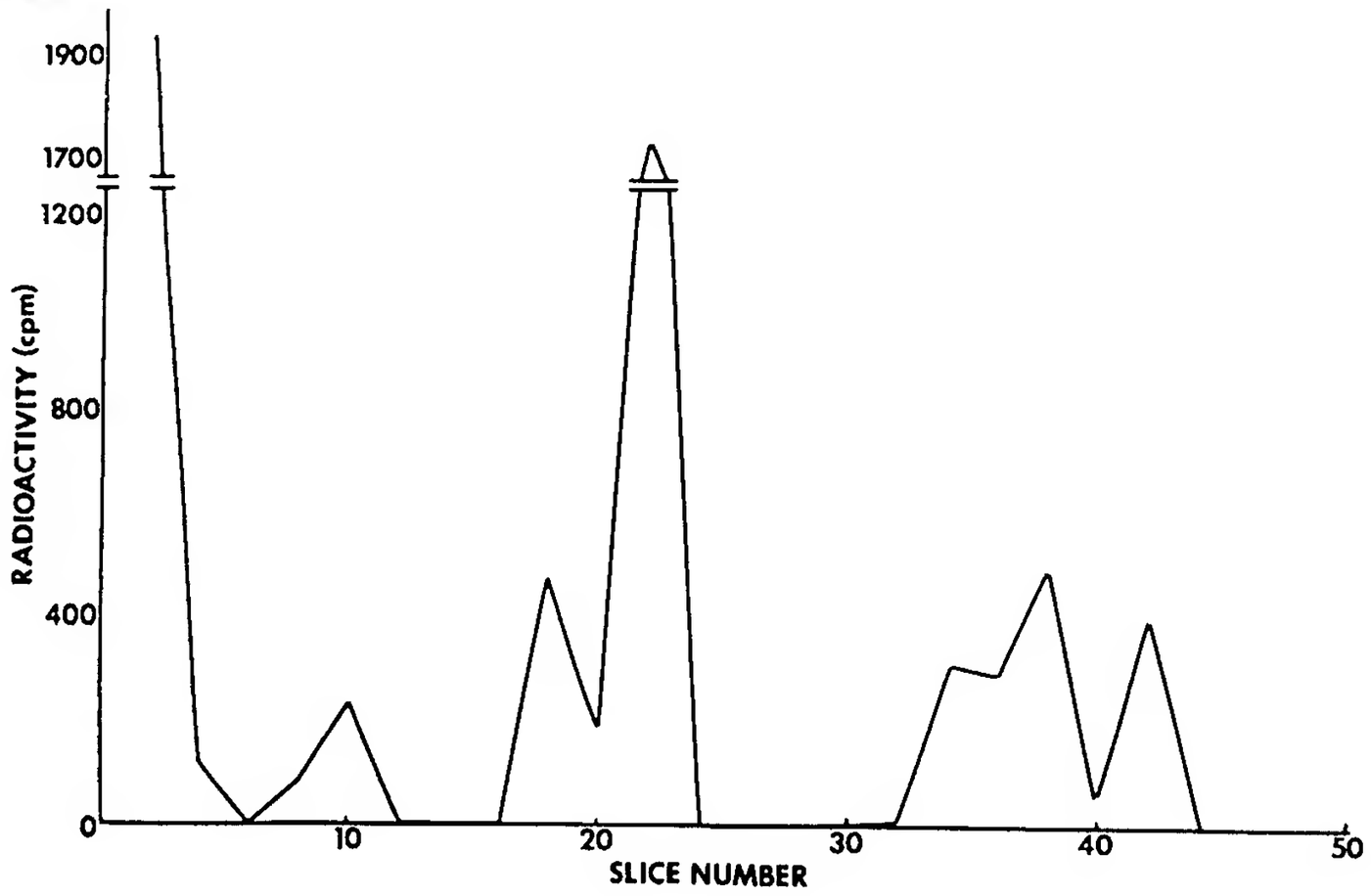

FIG. 3. Electrophoretic analysis demonstrating the heterogeneity of the protein kinase activity present in pooled fraction V. Pool V was collected and concentrated as described in the table, dialyzed against 0.13 *M* Tris · HCl, pH 8.1, and then electrophoresed in a 7.5% acrylamide gel as described in the text. The gel was sliced, each slice was eluted, and protein kinase assays were performed on the eluates in the presence of [γ-^{32}P]ATP as described in the text (no exogenous substrate was added). Although some activity is retained at the very top of the gel, the results show at least two major regions of protein kinase activity which migrate into the gel, thus demonstrating the multiplicity of protein kinases present even within a single pooled fraction (V).

them to become inactivated by other components in the relatively crude starting material usually used in such studies. Other investigators also routinely employ exogenous substrates, such as casein and histone, when assaying for protein kinase, and we have found that some of our enzyme activities are actually inhibited by these substrates. Thus, the present methodology has several distinct advantages over the techniques previously used, and although the exact extent of the multiplicity of nuclear protein kinases is not yet known, the present results indicate the existence of a large number of distinctly different enzymes involved in the phosphorylation of nonhistone chromatin proteins.

[17] Circular Dichroism Analysis of Nucleoprotein Complexes

By THOMAS E. WAGNER, VAUGHN VANDEGRIFT, and DEXTER S. MOORE

Although most spectroscopic methods (e.g., infrared, nuclear magnetic resonance, fluorescence) are particularly sensitive to the atomic composition of chemical groups and only grossly affected by the geometric arrangement of these groups or the geometry of their environment, optical activity spectroscopy is uniquely sensitive to these geometric aspects of molecular structure. For this reason, optical activity spectroscopy is the spectral method of choice for studies of molecular geometry.

One of the most biologically significant and interesting complexes is the eukaryote chromosome. In conjunction with genetic activity studies and chemical studies of this structure, much interest has been demonstrated recently in studies of the geometry or conformation of chromatin and simpler nucleoprotein complexes. The recent application of optical activity spectroscopy to the study of nucleoprotein conformation is now of sufficient interest to warrant a detailed descriptive discussion of this method and its specific application to the study of nucleoproteins.

The following sections are presented in order to acquaint researchers in the field of nucleoprotein chemistry and biology with some aspects of optical activity theory and instrumentation as well as a discussion of the analysis of nucleoprotein optical activity spectra.

Theory

Although optical activity spectroscopy began with optical rotatory dispersion measurements, this spectral method has been largely replaced by the more sensitive and reliable method of circular dichroism. An understanding of circular dichroism spectroscopy requires both a knowledge of the polarized energy used and the nature of its interaction with matter.

Plane-Polarized Light

Light energy is considered to consist of radiation of periodically varying electric and magnetic fields whose behavior is described by Maxwell's equations for an electromagnetic field. The electric and magnetic fields of this electromagnetic radiation oscillate at right angles to each other in a plane perpendicular to the direction of propagation of the light beam:

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Part E

Nuclear Structure and Function

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Table of Contents

EXHIBIT 2

[41] Assay of Cyclic AMP-Dependent Protein Kinases

By JACKIE D. CORBIN and ERWIN M. REIMANN

$$\text{Protein} + \text{ATP} \rightarrow \text{protein-P} + \text{ADP}$$

Assay Method

Principle. Most tissues contain protein kinases which are stimulated several-fold by cyclic 3′,5′-adenosine monophosphate (cAMP) and catalyze the transfer of phosphate from ATP to several proteins.[1] Incorporation of phosphate into protein can be monitored by transfer of ^{32}P to protein from [γ-^{32}P]ATP in the presence of magnesium. The phosphorylated protein is separated from the labeled precursor by adsorption of the precipitated protein on filter paper disks and washing the disks as described by several investigators.[2-5] Several proteins may be used as substrates in the assay, including muscle phosphorylase kinase[6] and glycogen synthetase,[7,8] protamine,[9] adipose tissue lipase,[10] casein,[6] specific histones,[11] and histone mixtures. When either phosphorylase kinase, glycogen synthetase, or lipase are used, the phosphorylation causes enzymatic activity changes which may be developed into alternate methods for assay of protein kinases.[6-8,10] In practice, however, such assays are difficult to quantitate. A histone mixture is a suitable substrate for several reasons: (1) it is available from commercial sources, (2) there is little if any protein kinase contamination, (3) an adequate amount of phosphate is incorporated, (4) it is a stable and easily precipitable protein mixture, and (5) the degree of stimulation of histone phosphorylation by cAMP is usually relatively high.

[1] E. G. Krebs, *Curr. Top. Cell. Regul.* **5**, 99 (1972).
[2] F. J. Bollum, *J. Biol. Chem.* **234**, 2733 (1959).
[3] R. J. Mars and G. D. Novelli, *Arch. Biochem. Biophys.* **94**, 48 (1961).
[4] W. B. Wastila, J. T. Stull, S. E. Mayer, and D. A. Walsh, *J. Biol. Chem.* **246**, 1996 (1971).
[5] E. M. Reimann, D. A. Walsh, and E. G. Krebs, *J. Biol. Chem.* **246**, 1986 (1971).
[6] D. A. Walsh, J. P. Perkins, and E. G. Krebs, *J. Biol. Chem.* **243**, 3763 (1968).
[7] D. L. Friedman and J. Larner, *Biochemistry* **4**, 2261 (1965).
[8] T. R. Soderling, J. P. Hickenbottom, E. M. Reimann, F. L. Hunkeler, D. A. Walsh, and E. G. Krebs, *J. Biol. Chem.* **245**, 6317 (1970).
[9] B. Jergil and G. H. Dixon, *J. Biol. Chem.* **245**, 425 (1970).
[10] J. K. Huttunen, D. Steinberg, and S. E. Mayer, *Biochem. Biophys. Res. Commun.* **41**, 1350 (1970).
[11] T. A. Langan, *Science* **162**, 579 (1968).

Reagents

Potassium phosphate, 50 m*M*, pH 6.8, with or without 6 μM cAMP
Histone mixture (type II-A, Sigma Chemical Company), 30 mg/ml in H_2O
Mg[γ-^{32}P]ATP (18 m*M* magnesium acetate, 1 m*M* [γ-^{32}P]ATP) in H_2O. Specific activity ~100 cpm/pmole. The [γ-^{32}P]ATP can be obtained from commercial sources or prepared by modification of the method of Glynn and Chappell[12,13]
Enzyme solution, 25–1000 units/ml
Trichloroacetic acid, 10%
Ethanol, 95%
Ethyl ether

Procedure. Equal volumes of the potassium phosphate, histone, and Mg[^{32}P]ATP are combined. Two mixtures, one with and one without cAMP, are usually prepared. The mixtures are stable for several weeks with repeated freezing and thawing. To disposable glass test tubes (1.2 × 7.5 cm) are added 50 μl of the mixtures. The reactions are initiated by pipetting 20 μl of the appropriate buffer (blank) or enzyme solution into the mixtures, mixing, and placing the reaction tubes in a water bath at 30°. After incubation 10 minutes, 50 μl of the reaction mixtures are spotted either on filter paper squares (Whatman 31 ET, 2 × 2 cm) or disks (Whatman 3 MM, 2.3 cm in diameter) numbered with pencil lead. The disks are dropped immediately into a screen wire basket located in a beaker containing ice cold 10% trichloroacetic acid (see Fig. 1). The screen basket is constructed so that ample space is provided for a magnetic stirring bar underneath the basket. The beaker should contain approximately 5 ml of trichloroacetic acid per filter disk. The filter disks are washed for 15 minutes in an ice bath at the slowest possible rotating speed of the stirring bar. The screen basket containing the filter disks is then removed from the beaker with forceps, and the trichloroacetic acid is poured into an appropriate radioactive waste container and replaced with fresh 10% trichloroacetic acid. This wash procedure is repeated three times at room temperature (total = 4 washes) for 15 minutes each. The filter disks are then washed in 95% ethanol and then ethyl ether for 5 minutes each. The disks are dried for approximately 5 minutes with a hair dryer, and then placed into a toluene-based scintillant for counting. Since the radioactive protein remains adsorbed to the filter disks during counting, the disks can be removed and the scintillant reused following counting. The last two trichloroacetic acid washes and the ethanol and ether washes can also be saved and reused. In addition to protecting the papers against damage by the stirring bar, the basket provides a convenient way of handling the papers during the washing and drying procedure. Stirring is normally included to ensure efficient washing, although we have found that low blanks can be obtained even without stirring. The blank is normally equivalent to 10 pmoles or less.

[12] I. M. Glynn, and J. B. Chappell, *Biochem. J.* **90**, 147 (1964).
[13] D. A. Walsh, J. P. Perkins, C. O. Brostrom, E. S. Ho, and E. G. Krebs, *J. Biol. Chem.* **246**, 1968 (1971).

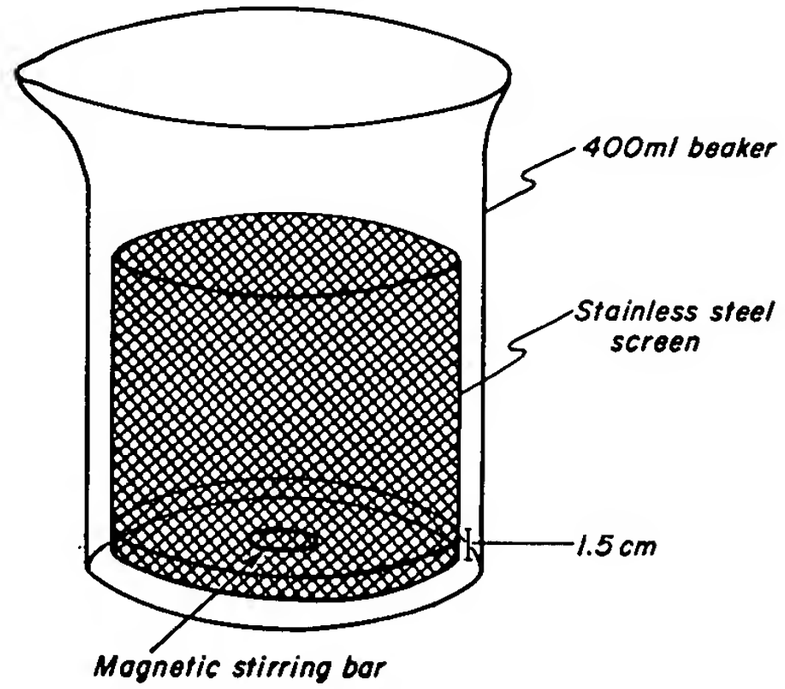

FIG. 1. Apparatus used for washing filter paper disks.

An alternate wash procedure to the filter disk method is that described by Walsh *et al.*[13] The filter disk method is preferable because it is less cumbersome, particularly when large numbers of samples (>25) are assayed.

The concentration of ATP can be reduced 10- to 100-fold, but this will reduce the reaction rate, increase substrate depletion by contaminating ATPases, and enhance the degree of inhibition by adenine nucleotides.[14]

Application of Assay Method to Crude Preparations. The procedure described above is suitable for assay of the enzyme in crude extracts of homogenates (2–30 ml/g). Dilute Tris or phosphate buffers containing 1 m*M* EDTA are appropriate homogenizing media. The ATPase activity of extracts of some tissues can be inhibitory. The inclusion of 40 m*M* NaF in the assay inhibits the ATPase activity in extracts of adipose tissue[15] but may not be necessary in other tissue extracts, such as heart

[14] H. Iwai, M. Inamasu, and S. Takeyama, *Biochem. Biophys. Res. Commun.* **46**, 824 (1972).
[15] J. D. Corbin, T. R. Sodering, and C. R. Park, *J. Biol. Chem.* **248**, 1813 (1973).

and skeletal muscle.[16] Because crude enzyme preparations may contain large amounts of endogenous substrates, there may be substantial phosphorylation without added substrate and it may be necessary to correct for this endogenous phosphorylation. If this is the case, two blanks [(1) no enzyme, (2) no substrate] are necessary.

Definition of Unit. One unit of protein kinase activity is that amount catalyzing transfer of 1 pmole of phosphate from [^{32}P]ATP to a histone mixture in 1 minute at 30°.

[16] T. R. Soderling, S. L. Keely, and J. D. Corbin, unpublished observations, 1972.

[42] Criteria for the Classification of Protein Kinases[1]

By J. A. Traugh, C. D. Ashby, and D. A. Walsh

Protein kinases catalyze the transfer of the terminal phosphate moiety of ATP to a variety of different protein substrates. Initially[2] the classification of these enzymes was, in accord with standard enzymological nomenclature, based on specificity with respect to the protein substrate. More recently it has become recognized that cyclic AMP (cAMP) regulates the activity of some, but not all, of these enzymes,[3,4] and from experimentation to date it would appear that many cAMP-regulated protein kinases exhibit a broad protein substrate specificity.[5] Thus, classical nomenclature becomes inoperable at the experimental level, although in the future cAMP-regulated protein kinases of unique protein substrate specificity may be recognized. The activation of cAMP-regulated protein kinases occurs by a cyclic nucleotide-promoted dissociation of the holoenzyme (designated RC) to yield the active catalytic species (C) and a complex consisting of regulatory subunit (R) and cAMP.[6-9] This reaction is shown in Eq. (1).

$$RC + cAMP \rightleftharpoons R \cdot cAMP + C \qquad (1)$$

[1] Supported by research grants Am 13618 from the U.S. Public Health Service. J.A.T. is a recipient of a Public Health Service Fellowship (GM 505590). D.A.W. is an Established Investigator of the American Heart Association.

[2] M. Rabinowitz *in* "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.) 2nd ed., Vol. 6, p. 119. Academic Press, New York, 1960.

[3] D. A. Walsh, J. P. Perkins, and E. G. Krebs, *J. Biol. Chem.* **243**, 376 (1968).

[4] J. F. Kuo and P. Greengard, *J. Biol. Chem.* **245**, 2493 (1970).

[5] For review see D. A. Walsh and E. G. Krebs *in* "The Enzymes" (P. D. Boyer, ed.), Vol. 8, p. 555. Academic Press, New York, 1973.

[6] M. A. Brostrom, E. M. Reimann, D. A. Walsh, and E. G. Krebs, *Advan. Enzyme Regul.* **8**, 191 (1970).

[7] M. Tao, M. L. Salas, and F. Lipmann, *Proc. Nat. Acad. Sci. U.S.* **67**, 408 (1970).

[8] G. N. Gill and L. D. Garren, *Biochem. Biophys. Res. Commun.* **39**, 335 (1970).

[9] A. Kumon, H. Yamamura, and Y. Nishizuka, *Biochem. Biophys. Res. Commun.* **41**, 1290 (1970).

Experimentally, the holoenzyme (RC) has been designated a cAMP-dependent protein kinase since the phosphotransferase activity is expressed only in the presence of cAMP.[10] From a physiological standpoint, free catalytic subunit (C) should also be considered as a cAMP-regulated protein kinase in that the activity in the cell is modulated in response to cAMP (by combination with R); nevertheless, the activity of the isolated enzyme (i.e., C) is expressed in the absence of the cyclic nucleotide.

In experimental situations, three categories of protein kinase can be recognized. Type *I*, holoenzyme (RC), is generally referred to as cAMP dependent protein kinase; type II is free catalytic subunit; and type III includes other protein kinases, the activity of which are not regulated by cAMP either *in vivo* or *in vitro*.

The purpose of this article is to present simple criteria that can be used experimentally to distinguish between the three categories of protein kinase. Of particular importance is the quantitative distinction between the enzymes designated types II and III, the activities of both of which are expressed in the absence of added cyclic nucleotide.

Physiologically, it is to be anticipated that free catalytic subunit will be formed in tissues in response to a hormonal stimulus, and that the amount present in a tissue homogenate will reflect the prior treatment of that tissue. Alternatively, free catalytic subunit may also occur by dissociation of the holoenzyme during the manipulations of extraction and purification. As studies of the effect of hormones on protein kinase dissociation *in vivo* become more extensive (see this volume [49]), it will become increasingly important to quantitate what amount of the activity expressed in the absence of cAMP represents free catalytic subunit. The criteria established here can be used quantitatively to distinguish the species of protein kinase in crude tissue extracts or, alternatively, to recognize the type of protein kinase in the purified enzyme preparations. Whereas these criteria cannot be conceived of as absolute, they are presented as working experimental guidelines.

Criteria for Classification

Four criteria may be used for the classification of various types of the protein kinases: stimulation of enzymatic activity by cAMP; binding of cAMP; inhibition by free regulatory subunit; and inhibition by the

[10] Although the holoenzyme has been designated cAMP dependent, it has not been demonstrated that this form is completely devoid of enzymatic activity in the absence of cAMP. Highly purified preparations of holoenzyme usually do exhibit some enzymatic activity in the absence of cyclic nucleotide, but it remains to be established whether this is due to holoenzyme per se or to free catalytic subunit produced by dissociation of holoenzyme at low protein concentration.

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Part C

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Table of Contents

Section I. Extraction and Purification of Cyclic Nucleotides

Section II. Assay of Cyclic Nucleotides

Product	Cat. No.			

Protein Kinase Substrates

Protein Kinase Substrate Peptide (Kemptide)

Protein Kinase Substrate Peptide (Kemptide)	**13119-011**	**5 mg**	**each**	**$100**

Protein Kinase Substrate Peptide (Kemptide) is a heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, MW = 771) protein kinase substrate (1,2). It has proven useful in routine assay (3) and kinetic analysis of cAMP-dependent protein kinase (PKA) (4). Its K_m for PKA has been reported as 4.7 μM (4).

Applications: This reagent has been used in biochemical investigations of protein kinases.

Performance and quality testing: Kemptide is determined to be >97% pure by HPLC. Identity is confirmed by amino acid analysis and functionality is verified in PKA assays.

Recommended storage condition: −20°C.

1. Kemp, B.E., Graves, D.A., and Krebs, E.G. (1976) *Fed. Proc. 35,* 1384
2. Maller, J.L., Kemp, B.E., and Krebs, E.G. (1978) *Proc. Natl. Acad. Sci. USA 75,* 248.
3. Roskoski, R., Jr., (1983) *Methods Enzymol. 99,* 3.
4. Whitehouse, S., Feramisco, J.R., Casnellie, J.E., Krebs, E.G., and Walsh, D.A. (1983) *J. Biol. Chem. 258,* 3693.

Protein Kinase C Substrate Peptide [Ser^{25}]PKC(19–31)

Protein Kinase C Substrate Peptide [Ser^{25}]PKC(19–31)	**13121-017**	**1 mg**	**each**	**$110**

Protein Kinase C Substrate Peptide [Ser^{25}]PKC(19–31) is a derivative of a 13-amino-acid segment of Protein Kinase C (PKC) in which the alanine corresponding to position 25 in PKC has been replaced with serine, producing an excellent substrate for PKC (1). The sequence of this peptide (MW = 1,558) is Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val. As a PKC substrate, [Ser^{25}]PKC(19–31) exhibits a K_m of 0.2 μM (1), which is lower than the K_m reported for other PKC peptide substrates (2).

Applications: [Ser^{25}]PKC(19–31) is useful for biochemical studies of PKC, or in place of histone for routine assay of PKC activity.

Performance and quality testing: [Ser^{25}]PKC(19–31) is determined to be >97% pure by HPLC. Identity is confirmed by amino acid analysis, and functionality is verified by use in PKC assays.

Recommended storage condition: 4°C.

1. House, C. and Kemp, B.E. (1987) *Science 238,* 1726.
2. House, C., Wettenhall, R.E.H., and Kemp, B.E. (1987) *J. Biol. Chem. 262,* 772.

NOTE: Old and new worldwide catalogue numbers are cross-referenced in the catalogue number indexes beginning on page NI-1.

Product	Cat. No.	Size	Qty.

Protein Kinase C Substrate Peptide Ac-MBP(4–14)

Protein Kinase C Substrate Peptide Ac-MBP(4–14)	13127-014	1 mg	each

Protein Kinase C Substrate Peptide Ac-MBP(4–14) is a synthetic peptide based myelin basic protein sequence and acetylated at the N-terminal glutamine to in its stability. It can act as a specific substrate for protein kinase C (PKC). The sequence of this peptide (MW = 1,433) is Ac-Gln-Lys-Arg-Pro-Ser-Gln-Arg-Se Tyr-Leu. Acetylation has no significant effect on the ability of the peptide to ac PKC substrate: using PKC from HL-60 cells, the K_m of Ac-MBP(4–14) is 5 μM compared to 7 μM as reported by Yasuda *et al.* for unacetylated MBP(4–14) (1

Applications: Ac-MBP(4–14) is a useful substrate for routine assay of PKC activ is the substrate provided in the Protein Kinase C Assay System). MBP(4–14) h specifically been used in the assay of PKC from rat brain (1) and from HL-60 c

Performance and quality testing: Ac-MBP(4–14) is determined to be >98% p HPLC. Identity is confirmed by amino acid analysis, and functionality is ve a PKC assay.

Recommended storage condition: 4°C.

1. Yasuda, I., Kishimoto, A., Tanaka, S., Tominaga, M., Sakurai, A., and Nishizuka (1990) *Biochem. Biophys. Res. Commun. 166,* 1220.
2. Hashimoto, K., Kishimoto, A., Aihara, H., Yasuda, I., Mikawa, K., and Nishizuk (1990) *FEBS Lett. 263,* 31.

See also:

Anti-Protein Kinase C Antibodies, pages 16-5 to 16-7.
Phorbol Esters, page 16-25.
Protein Kinase C products, pages 16-2 to 16-20.

Protein Kinase C Substrate Peptide [Ala^{9,10},Lys^{11,12}]GS(1–12)

Protein Kinase C Substrate Peptide [Ala^{9,10},Lys^{11,12}]GS(1–12)	13129-010	1 mg	each

Protein Kinase C Substrate Peptide [Ala^{9,10},Lys^{11,12}]GS(1–12) is a synthetic peptide derived from the sequence of glycogen synthase, a physiologically substrate for protein kinase C (PKC) (1,2). This peptide is prepared by repl serines with alanines at positions 9 and 10 of GS(1–10) and adding two C-te lysine residues (1). The sequence of this peptide (MW = 1,270) is Pro-Leu-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys. [Ala^{9,10},Lys^{11,12}]GS(1–12) exhibits K_m of 4.1 μM for PKC (1). It is also a good substrate for the multifunction Ca^{2+}/CaM-dependent protein kinase (CaMKII), with a K_m of 18 μM (1).

Applications: [Ala^{9,10},Lys^{11,12}]GS(1–12) is a useful substrate for routine ass PKC activity. It has been used in the study of PKC substrate requirements activation (3), and "pseudosubstrate" inhibition (4).

Performance and quality testing: [Ala^{9,10},Lys^{11,12}]GS(1–12) is determined to >98% pure by HPLC. Identity is confirmed by amino acid analysis, and functionality is verified in a PKC assay (5).

Recommended storage condition: 4°C.

1. House, C., Wettenhall, R.E.H., and Kemp, B.E. (1987) *J. Biol. Chem. 262,* 772.
2. Ahmad, Z., Lee, F.-T., DePaoli-Roach, A., and Roach, P.J. (1984) *J. Biol. Chem. 259,* 8743.
3. House, C., Robinson, P.J., and Kemp, B.E. (1989) *FEBS Lett. 249,* 243.
4. House, C. and Kemp, B.E. (1987) *Science 238,* 1762.
5. Bell, R.M., Hannun, Y., and Loomis, C. (1986) *Methods Enzymol. 124,* 353.

See also:

Anti-Protein Kinase C Antibodies, pages 16-5 and 16-7.
Phorbol Esters, page 16-25.
Protein Kinase C products, pages 16-2 to 16-20.

Product	Cat. No.	Size	Qty.	Price

Protein Tyrosine Kinase Substrate Peptide RR-SRC

Protein Tyrosine Kinase Substrate Peptide RR-SRC	13124-011	1 mg	each	$75

Protein Tyrosine Kinase Substrate Peptide RR-SRC is a synthetic 13-amino-acid peptide derived from the amino acid sequence surrounding the phosphorylation site in $pp60^{src}$ (1). The sequence of this peptide (MW = 1,520) is Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly; the inclusion of two arginine residues at the amino terminus enables this substrate to bind to phosphocellulose. This peptide was originally tested as a substrate for the tyrosine kinase activity associated with the EGF receptor (EGF-R) (2), with a K_m of 0.5 mM (3).

Performance and quality testing: RR-SRC is determined to be >97% pure by HPLC. Identity is confirmed by amino acid analysis, and functionality is verified in a tyrosine kinase assay using membrane preparations of A431 epidermoid carcinoma cells (EGF-R activity) and purified $p43^{v\text{-}abl}$ kinase.

Recommended storage condition: 4°C.

1. Czernilofsky, A.P., Levinson, A.D., Varmus, H.E., Bishop, J.M., Tischer, E., and Goodman, H.M. (1980) *Nature (Lond.) 287,* 198.
2. Pike, L.J., Gallis, B., Casnellie, J.E., Bornstein, P., and Krebs, E.G. (1982) *Proc. Natl. Acad. Sci. USA 79,* 1443.
3. Casnellie, J.E., Harrison, M.L., Pike, L.J., Hellstrom, K.E., and Krebs, E.G. (1982) *Proc. Natl. Acad. Sci. USA 79,* 282.

See also:

Anti-Phosphotyrosine Monoclonal Antibody 6G9 (Cat. No. 33160-011), page 16-8.
Protein Tyrosine Kinase Assay System (Cat. No. 13154-018), page 16-3.
Protein Tyrosine Kinase Inhibitors, pages 16-21 to 16-25.

Casein Kinase II Substrate Peptide

Casein Kinase II Substrate Peptide	13126-016	1 mg	each	$110

Casein Kinase II Substrate Peptide is a synthetic 10-amino-acid peptide that acts as a specific substrate for casein kinase II (CKII) (1), a widely distributed serine/threonine protein kinase (2) that requires acidic amino acids surrounding substrate phosphorylation sites (1,3). The sequence of this peptide (MW = 1,362) is Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu. This substrate has a relatively high K_m for CKII (0.5 mM) (1) but is highly specific: cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase, phosphorylase kinase, smooth muscle myosin light chain kinase, insulin- and EGF-stimulated kinases, and casein kinase I are all unable to use this peptide as a substrate (1).

Performance and quality testing: CKII Substrate Peptide is determined to be >97% pure by HPLC. Identity is confirmed by amino acid analysis, and functionality is verified in CKII assays using A431 cell extracts.

Recommended storage condition: 4°C.

1. Kuenzel, E.A. and Krebs, E.G. (1985) *Proc. Natl. Acad. Sci. USA 82,* 737.
2. Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. (1987) *Annu. Rev. Biochem. 56,* 567.
3. Kuenzel, E.A., Mulligan, J.A., Sommercorn, J., and Krebs, E.G. (1987) *J. Biol. Chem. 262,* 9136.

NOTE: Old and new worldwide catalogue numbers are cross-referenced in the catalogue number indexes beginning on page NI-1.

Product	Cat. No.	Size	Qty.

Myelin Basic Protein

Myelin Basic Protein	13228-010	10 mg	each

Myelin Basic Protein (MBP) was purified from bovine brain by a modification of the method of Deibler *et al.* (1). MBP is an excellent *in vitro* substrate for a number of protein kinases and phosphatases including protein kinase C, cAMP-dependent protein kinase (2), Ca^{2+}/calmodulin-dependent protein kinase II (3), MAP2 kinases (4,5), and protein tyrosine phosphatase CD45 (7). Cyanogen bromide cleavage of this preparation of MBP gave results consistent with identity as the 18.5-kDa form of MBP (8–10). The protein is supplied lyophilized.

Performance and quality testing: The product is determined to be ≥90% pure by SDS-PAGE.

Recommended storage condition: −20°C.

1. Deibler, G.E., Martenson, R.E., and Kies, M.W. (1972) *Prep. Biochem. 2,* 139.
2. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985) *J. Biol. Chem. 260,* 12492.
3. Hanson, P.I. and Schulman, H. (1992) *Annu. Rev. Biochem. 61,* 559.
4. Erickson, A.K., Payne, D.M., Martino, P.A., Rossomando, A.J., Shabanowitz, J., Weber, M.J., Hunt, D.F., and Sturgill, T.W. (1990) *J. Biol. Chem. 265,* 19728.
5. Clark-Lewis, I., Sanghera, J.S., and Pelech, S.L. (1991) *J. Biol. Chem. 266,* 15180.
6. Tonks, N.K., Diltz, C.D., and Fischer, E.H. (1991) *Meth. Enzymol. 201,* 442.
7. Eylar, E.H., Brostoff, S., Hashim, G., Caccam, J., and Burnett, P. (1971) *J. Biol. Chem. 246,* 5770.
8. Takahashi, N., Roach, A., Teplow, D.B., Prusiner, S.B., and Hood, L. (1985) *Cell* 139.

See also:

Protein Kinase Substrate Peptides, pages 16-13 to 16-18.
RCM-Lysozyme (Cat. No. 13229-018), page 16-16.

RCM-Lysozyme

RCM-Lysozyme	13229-018	10 mg	each

RCM-Lysozyme (Reduced Carboxamidomethylated and Maleylated Lysozyme) is a standard substrate for protein tyrosine phosphatases (1). The substrate can be ^{32}P-labeled by a variety of protein tyrosine kinases. Phosphorylation on a single tyrosine residue (tyr 53) can be achieved at relatively high stoichiometry (0.2–0.5 mol/mol) by crude membrane preparations of the EGF/insulin receptor (2). Chemical modification of lysozyme is necessary for phosphorylation/dephosphorylation to occur. Reduction of lysozyme cleaves the four disulfide bridges in the molecule exposing tyr 53, while carboxamidomethylation of the thiol group prevents refolding. Maleylation of lysine residues improves the solubility of the protein so it remains in solution throughout phosphorylation and dephosphorylation procedures.

Applications: RCM-Lysozyme can be used as a substrate for protein tyrosine kinases (2) and protein tyrosine phosphatases (2) *in vitro.* ^{32}P-labeled RCM-Lysozyme has been microinjected into oocytes to measure PTPase activity *in vivo* (3).

Performance and quality testing: The product is determined to be ≥95% pure by SDS PAGE. The substrate is identified by SDS-PAGE.

Recommended storage condition: −20°C, dessicated.

1. Tonks, N.K., Dlitz, C.D., and Fischer, E.H. (1988) *J. Biol. Chem. 263,* 6722.
2. Tonks, N.K., Dlitz, C.D., and Fischer, E.H. (1991) *Meth. Enzymol. 201,* 427.
3. Cicirelli, M.F., Tonks, N.K., Dlitz, C.D., Fischer, E.H., and Krebs, E.G. (1990) *Proc. Natl. Acad. Sci. USA 87,* 5514.

See also:

Anti-Phosphotyrosine Rabbit Polyclonal Antibody (Cat. No. 13216-015), page 16-7.
Myelin Basic Protein (Cat. No. 13228-010), page 16-16.
Protein Kinase Substrate Peptides, pages 16-13 to 16-18.
Protein Tyrosine Kinase Assay System (Cat. No. 13154-018), page 16-3.

Product	Cat. No.	Size	Qty.	Price

Histone H1

Histone H1 **13221-015** **10 mg** **each** **$55**

Histone H1 is purified from calf thymus by the method of Cole (1). The Histone H1 class consists of several H1 variants that represent either different isoforms of H1 or various post-translational modifications, only some of which can be distinguished electrophoretically (2). It migrates on SDS polyacrylamide gels as a tight group of bands with an apparent Mr of approximately 33 kDa, although its true size is recognized to be 21 kDa (2).

Applications: Histone H1 is commonly used as a substrate for a number of protein kinases (3) and protein phosphatases (4), as well as for chromosome-reconstitution studies (2).

Performance and quality testing: Histone H1 is determined to be >95% pure by SDS-PAGE.

Recommended storage condition: −20°C.

1. Cole, R.D. (1989) *Meth. Enzymol. 170*, 524.
2. Cole, R.D. (1987) *Int. J. Peptide Protein Res. 30*, 433.
3. Cicirelli, M.F., Pelech, S.L., and Krebs, E.G. (1988) *J. Biol. Chem. 263*, 2009.
4. Suganuma, M., Fujiki, H., Furya-Suguri, H., Yoshizawa, S., Yasumoto, S., Kato, Y., Fusetani, N., and Sugimura, T. (1990) *Cancer Res. 50*, 3521.

See also:

Protein Kinase Substrate Peptides, pages 16-13 to 16-18.

$p34^{cdc2}$ Peptide (PSTAIR)

$p34^{cdc2}$ Peptide (PSTAIR) **13385-018** **1 mg** **each** **$125**

$p34^{cdc2}$ Peptide (PSTAIR) is a synthetic 16-amino-acid peptide whose sequence is perfectly conserved in cell cycle regulatory kinases from a wide variety of organisms. These include the cdc2 kinase of fission yeast and its homologues in budding yeast (CDC28), humans ($p34^{cdc2}$), and the p34 kinase component of the maturation-promoting factor (MPF) of starfish and frog oocytes (1). The sequence of this peptide (MW = 1,742) is Glu-Gly-Val-Pro-Ser-Thr-Ala-Ile-Arg-Glu-Ile-Ser-Leu-Leu-Lys-Glu. This preparation of PSTAIR is supplied lyophilized.

Performance and quality testing: PSTAIR is determined to be >97% pure by HPLC. Identity is confirmed by amino acid analysis.

Recommended storage condition: 4°C.

1. Norbury, C.J. and Nurse, P. (1989) *Biochim. Biophys. Acta 989,* 85.

NOTE: Old and new worldwide catalogue numbers are cross-referenced in the catalogue number indexes beginning on page NI-1.

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fact, the lower detection limit in this assay is determined by the level of background phosphorylation and is therefore variable. These differences are probably the main reason for the relatively high sensitivity and accuracy of the nonradioactive assay presented here. Another advantage of the new method is the absence of restrictions in the choice of the ATP concentration in the assay mixture. In the conventional filter-paper assay the choice of the ATP concentration is a compromise between the need for a high specific activity of the [^{32}P]ATP and the amount of radioactivity which can be handled at the same time. As a consequence the ATP concentration is usually rather low, thus underestimating the activity of higher K_m PTKs and limiting the time in which the reaction is linear. Finally, the main advantage of the new method is probably the avoidance of the use of radioactivity, which enables the simultaneous assay of a large number of samples without the need for special facilities and safety precautions. Therefore, this method seems to be useful to facilitate further studies on PTKs from various sources and, moreover, makes the PTK activity determination more readily accessible for routine diagnostic purposes.

Method B: Solid-Phase Assay System

1. Pretreat a PVDF membrane according to the procedure described in Method A.
2. Spot 5-μl aliquots of PTK samples directly on the membrane and allow the spots to dry (do not let the membrane become too dry).
3. Incubate the membrane with PGT (2 mg/ml in incubation buffer, see Method A) for 30 min at room temperature.
4. Rinse the membrane for 10 sec in doubly distilled water or incubation buffer.
5. Incubate the membrane with ATP (500 μM in incubation buffer) for 60 min at 37°.
6. Proceed with the detection and quantification of phosphotyrosyl residues by the immunogold silver-staining procedure as described in Method A.

Evaluation of Method

Although the solid-phase variant of the PTK assay described here provides only semiquantitative results, it is very suitable when a rapid screening of many PTK samples is desired, for instance, in purification procedures. It should be noted, however, that the assay conditions are quite different from the liquid-phase assay: The PTK protein is adsorbed

EXHIBIT 4

to the membrane before the phosphorylation reaction is started and excess PGT, not bound to the enzyme or the membrane, is washed away before adding ATP as a second substrate. As a consequence PTK activities from the two assay systems cannot be compared directly. Some PTKs may be relatively inactive in the solid-phase assay, although they are very active in the liquid-phase assay or vice versa.

[7] Use of Synthetic Amino Acid Polymers for Assay of Protein-Tyrosine and Protein-Serine Kinases

By EFRAIM RACKER

Introduction

Synthetic random polymers of amino acids with an average molecular weight of about 30,000 to 60,000 serve as excellent substrates for many protein kinases (PK). A polymer of glutamate (E) and tyrosine (Y) with a ratio of 4 : 1 is a substrate for all protein-tyrosine kinases (PTK) thus far tested.[1-3] A large number of protein-serine kinases (PSK) phosphorylate either poly(arginine–serine), RS (3 : 1), or poly(glutamate–threonine), ET (4.4 : 1), at rates comparable to those observed with natural substrates.[4] Insertion of other amino acids into glutamate–tyrosine polymers such as alanine (A) or lysine (K) markedly alters the rates of phosphorylation catalyzed by different PTKs,[1] as will be described in the section, Substrate Specificity. Nevertheless, the fact that EY (4 : 1), RS (3 : 1), and ET (4.4 : 1) serve as excellent substrates for many protein kinases[1-4] raises the question of the significance of so-called "consensus sequences." Although convincing evidence for the importance of the amino acid sequences has been demonstrated in numerous laboratories with synthetic low-molecular-weight peptides as substrates,[5] the question of how much it contributes to the susceptibility of large proteins to phosphorylation remains to be established. For example, RAS 2 of yeast, lacking the classical consensus

[1] S. Braun, W. E. Raymond, and E. Racker, *J. Biol. Chem.* **259,** 2051 (1984).
[2] S. Braun, M. Abdel-Ghany, J. A. Lettieri, and E. Racker, *Arch. Biochem. Biophys.* **247,** 424 (1986).
[3] S. Nakamura, S. Braun, and E. Racker, *Arch. Biochem. Biophys.* **252,** 538 (1987).
[4] M. Abdel-Ghany, D. Raden, E. Racker, and E. Katchalski-Katzir, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 1408 (1988).
[5] P. D. Boyer and E. G. Krebs, eds., "The Enzymes," Vols. 17 and 18. Academic Press, Orlando, Florida, 1987.

sequence for protein kinase A (PK-A), is a better substrate for this enzyme than histone 1.[6] It was proposed[7,8] that charge distribution plays an important role in substrate susceptibility and that the distance between a tyrosine and a charged amino acid (glutamate or aspartate), or between serine and arginine or lysine, is a key signal for attack by protein kinases. An appropriate charge constellation may be provided by the primary sequence or induced by the three-dimensional conformation of proteins or even by the addition of charged polymers that are not substrates.[9,10] A remarkable feature of these polymer substrates, particularly in the case of PTK, is that their K_m values may be orders of magnitude lower than those of small consensus sequence peptides. A second advantage is that random polymers are easily synthesized and many are commercially available at a reasonable price. Moreover, a tyrosine polymer can be used as substrate in crude cell extracts without interference by the large number of protein-serine kinases. A disadvantage of the currently available polymers is that they are very heterogeneous with respect to molecular weight and therefore not very useful for SDS–PAGE analysis. They can be used, however, in gel electrophoresis in the absence of SDS.[11] Some basic polymers do not even enter the SDS–polyacrylamide gel. A most attractive feature is that the polymers can be used for studies of signal transduction between either tyrosine and serine kinases or between serine and tyrosine kinases, as will be described below.

Substrates

Synthetic random amino acid polymers are synthesized as described.[12] Several tyrosine-containing polymers, poly(arginine–serine), and poly(lysine–serine), can be purchased from Sigma (St. Louis, MO). They are widely used for the assay of protein kinases.[2–4,11,13–15]

[6] R. Resnick and E. Racker, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 2474 (1988).
[7] E. Racker, *JNCI, J. Natl. Cancer Inst.* **81,** 247 (1989).
[8] M. Abdel-Ghany, H. K. Kole, M. Abou El Saad, and E. Racker, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6072 (1989).
[9] E. Racker, *in* "Current Topics in Cellular Regulation." (G. R. Welch, ed.) Academic Press, in press.
[10] M. Abdel-Ghany, K. El-Gendy, S. Zhang, and E. Racker, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7061 (1990).
[11] G. Schieven, J. Thorner, and G. S. Martin, *Science* **231,** 390 (1986).
[12] M. Sela and L. A. Steiner, *Biochemistry* **2,** 416 (1963).
[13] N. Sasaki, R. W. Rees-Jones, Y. Zick, S. P. Nissley, and M. M. Rechler, *J. Biol. Chem.* **260,** 9793 (1985).
[14] K. Yonezawa and R. A. Roth, *FASEB J.* **4,** 194 (1990).
[15] Y. Yanagita, M. Abdel-Ghany, D. Raden, N. Nelson, and E. Racker, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 925 (1987).

Assays

Assays are performed in Eppendorf tubes in the presence of appropriate activators. For each protein kinase optimal conditions of buffer, pH, cations, reducing agents, activators, ATP, substrate concentration, and linear time course need to be established. With preparations that contain phosphatase activity, inhibitors are added (e.g., 100 μM vanadate) provided they do not interfere with the PK activity. Sometimes lowering the temperature to 4° is used to reduce the relative effectiveness of phosphatases and proteases. Optimal concentrations of the polymers are critical. The K_m values between polymers for, e.g., the v-p140 PTK of Fujinami virus vary between 6 μg and 1.5 mg/ml.[1] Amounts of polymer in excess of optimum sometimes inhibit PTK activities. However, optimal substrate concentrations may vary in the presence of charged activators. Protein kinase P (PK-P) is a membranous casein kinase similar to but not identical to casein kinase II.[15] PK-P phosphorylation of some protein substrates has an absolute requirement for poly(lysine). With the ET polymer, poly(lysine) shows only marginal stimulation of PK-P activity when optimal concentrations of the acidic polymers are used, but stimulation is pronounced in the presence of suboptimal concentrations of the polymer substrate.[4]

A typical experiment is performed in a final volume of 50 μl containing 20 mM HEPES buffer (pH 7.4), enzyme, polymer, 10 mM $MgCl_2$, and 10 μM [γ-^{32}P]ATP (specific radioactivity of 3000 to 8000 cpm/pmol). After incubation at room temperature (5 to 60 min), 30-μl samples are removed and placed on 2-cm filter paper squares (Whatman 3 MM), held by a forceps. Each square is immediately immersed into a plastic round container (~6 in. in diameter) containing about 50 ml of 10% trichloroacetic acid and 10 mM sodium pyrophosphate, resulting in the precipitation of the polymers. The container is closed and gently shaken for 1 to 2 hr (depending on background), changing the fluid every 15 min. After removal of the squares by forceps, they are dried in an oven or on a hot plate and counted in a scintillation counter.

Substrate Specificity

Tyrosine Polymers

There are distinct differences with respect to substrate specificity with various PTKs. For the insulin receptor PTK the best substrate is EY (4 : 1); less active is EAY (6 : 3 : 1). Most other polymers, including EKAY

(36 : 24 : 35 : 5), are less than 10% as active. The EGF receptor and v-*abl* PTKs prefer EAY (6 : 3 : 1) over EY (4 : 1) as substrate. For the v-*fps* PTK EKAY is by far the best substrate, whereas EY (1 : 1) is about 30% as active and is slightly superior to EY (4 : 1). The latter is a suitable substrate for all the other PTKs tested, including two enzymes from placenta, one from brain,[2] and one from Ehrlich ascites tumor cells.[3]

Of particular interest are the polymers EY (1 : 1) and EAY (1 : 1 : 1). EY (1 : 1) is a substrate for the v-*fps* PTK (1), but it is very poorly phosphorylated by the insulin and EGF receptor kinases. It was shown that EY (1 : 1) is an excellent substrate for insulin receptor PTK if large amounts of bovine serum albumin (BSA) (up to 10 mg/ml) are present.[14] We have shown that the same is true for c- and v-*src* kinases. Stimulation by BSA is most pronounced with EAY (1 : 1 : 1) as substrate.[10] From plasma membrane of bovine brain an activator was extracted and partially purified that stimulates phosphorylation of EY (4 : 1) by c-*src* kinase. Of special interest is that stimulation of v-*src* activity by this activator was small by comparison with c-*src*. The activation was separated from an inhibitor that preferentially inhibits c-*src*.[10]

These examples document again the phenomenon of chaperones or substrate modulators, proteins that are not kinase substrates themselves but greatly influence phosphorylation of other proteins.[7,8,9]

Use of Synthetic Amino Acid Polymers for Study of Signal Transduction between Protein Kinases

With the availability of synthetic polymers that contain either tyrosine or serine or threonine as the only amino acid susceptible to phosphorylation, the effect of a tyrosine kinase on serine kinase or of serine kinases on tyrosine kinases can be explored *in vitro*. However, it is essential to assay under conditions of appropriate kinetics to show cross-talk effects. As a rule the converter enzyme must be present in large excess, whereas the enzyme to be converted must be present at small concentrations within the linear range of activity and preferably with its substrate at saturating concentrations. Attention must be drawn to the fact that very acidic polymers, e.g., EY (4 : 1), may inhibit serine kinases, e.g., casein kinase II or PK-P, and basic polymers in excess may inhibit PTKs.[8] Activators of PK-C (Ca^{2+} and lipids) stimulate *src* PTK activity.[10] It is therefore essential that signal transduction experiments of this kind are performed in the same assay medium so that controls with single enzymes are meaningful.

With cell extracts or partially purified protein kinase preparations that contain multiple kinases, it would be of great advantage to have specific PK inhibitors available. Unfortunately, very few specific inhibitors are

generally available. In our hands, using synthetic polymers as substrate, only the peptide inhibitor of PK-A has proved to be a specific inhibitor. Staurosporine inhibits protein kinase C at 5 n*M* concentration without affecting several other PSKs. However, it is also a potent inhibitor of PTKs, e.g., src. Other inhibitors such as sphingosine[16] and H7, were found to lack specificity. Our knowledge of specific consensus sequences required for the phosphorylation of small polypeptides should, however, become very valuable for the design of specific kinase inhibitors and are slowly becoming available. However, in contrast to cell-permeable inhibitors, such as staurosporine, or activators, such as epinephrine or phorbol esters, these polypeptides are not suitable for experiments on signal transduction in intact cells and specific permeable inhibitors would be of great value.

Use of Synthetic Polymers for Purification of Protein Kinases

Affinity columns with synthetic polymers are easily prepared and useful for the purification of protein kinases. Several unidentified PTKs were prepared from human placenta, bovine brain,[2] and Ehrlich ascites tumors.[3] In some cases the extent of purification was modest with one polymer affinity column and much better with another. For example, in the case of c-src and v-src purification, two polymer columns used sequentially yielded a virtually homogeneous kinase protein.[17] On these affinity columns c-src and v-src kinases eluted at different salt concentrations. Exploration of the marked differences both in substrate specificity and K_m values described above should greatly increase the potential usefulness of such affinity columns.

Acknowledgments

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[16] Y. Igarashi, S. Hakomori, T. Toyokuni, B. Dean, S. Fujita, M. Sugimoto, T. Ogawa, K. El-Ghendy, and E. Racker, *Biochemistry* **28,** 6796 (1989).
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Table of Contents

Determination of Cyclic Nucleotide-Dependent Protein Kinase Substrate Specificity by the Use of Peptide Libraries on Cellulose Paper†

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ABSTRACT: An iterative approach to the a priori determination of the substrate specificity of cAMP- and cGMP-dependent protein kinases (PKA and PKG) by the use of peptide libraries on cellulose paper is described. The starting point of the investigation was an octamer library with the general structure Ac-XXX12XXX, where X represents mixtures of all 20 natural amino acids and 1 and 2 represent individual amino acid residues. The library thus contained all possible 2.56×10^{10} octamers, divided into 400 sublibraries with defined amino acids 1 and 2 each consisting of 6.4×10^7 sequences. After phosphorylation with the kinases in the presence of [γ-^{32}P]ATP, the sublibrarys Ac-XXXRRXXX and Ac-XXXRKXXX were identified as the best substrates for PKA and PKG, respectively. The second-generation libraries had the structures Ac-XXXRR12X and Ac-XXXRK12X for PKA and PKG and resulted in the most active sequence pools Ac-XXXRRASX and Ac-XXXRKKSX. After delineation of every position in the octameric sequence and extension of the investigation to decameric peptides, the best sequences, Ac-KRAERKASIY and Ac-TQKARKKSNA, were obtained for PKA and PKG, respectively. Promising octameric and decameric peptides were assembled 5 or 10 times each and assayed in order to determine the experimental scatter inherent in the approach. The kinetic data of several octameric and decameric sequences were determined in solution and compared to data for known substrates. The recognition motif of PKA was confirmed by this approach, and a novel substrate sequence for PKG was identified. The approach can be expected to be of generally applicable for the elucidation of protein kinase specificity with linear peptide substrates.

Protein phosphorylation by protein kinases is the most important regulatory mechanism of cellular function and signal transduction (Hunter, 1987). Among the superfamily of protein kinases the two cyclic nucleotide regulated protein kinases, cAMP-dependent protein kinase (PKA)[1] and cGMP-dependent protein kinase (PKG), form a closely related subfamily of serine/threonine kinases [for reviews, see Francis and Corbin (1994a), Hofmann et al. (1992), and Taylor et al. (1993)].

PKA and PKG share many structural features such as the N-terminal dimerization region (Landgraf & Hofmann, 1989; Landgraf et al., 1990; Zick & Taylor, 1986), a proteolytic sensitive hinge region which also contains the substrate recognition sequence, two in-tandem cyclic nucleotide binding sites which allosterically regulate the enzyme activity, and a catalytic subunit responsible for phosphorylating the substrate (Titani et al., 1982; Takio et al., 1984). Despite these similarities, the two enzymes display distinct differences which account for their unique properties. While the PKA holoenzyme complex dissociates upon activation into the regulatory subunit dimer and the active catalytic subunits, the PKG holoenzyme complex remains as an entity and does not dissociate (Francis & Corbin, 1994b; Hofmann et al., 1992).

An additional characteristic difference is the substrate specificity. In general, protein kinases exhibit specificities that are often primarily determined by the amino acids around the phosphorylation sites (Kemp & Pearson, 1991; Glass, 1983). While PKA displays a well-defined specificity with the consensus motif RRXS(A)X for substrate or inhibitory peptides (Glass et al., 1989), PKG seems to have a less well defined recognition sequence (Glass, 1990; Butt et al., 1994). However, identification of amino acids that contribute to substrate motifs is essential for developing specific peptide substrates and inhibitors. In this respect, many investigations with large numbers of individual peptides have been conducted in order to find high-affinity substrates as well as inhibitors. Peptide libraries offer the possibility of investigating the sequence dependence of the phosphorylation more thoroughly and systematically. Recently, two approaches have been described that utilized peptide libraries generated by the method of "split synthesis" on resins (Till et al. 1994; Wu et al. 1994).

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[1] Abbreviations: Standard one-letter and three-letter abbreviations of amino acids have been used. Ac, acetyl; Acm, acetamidomethyl; BSA, bovine serum albumin; DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 1-fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; PKA, catalytic subunit C_α of the cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; RP, reversed phase; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid.

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By employing cAMP- and cGMP-dependent protein kinases as model enzymes, we decribe here a new method for the systematic investigation of the sequence-dependent specificity of protein kinases with peptide libraries on cellulose paper. We followed an iterative approach similar to the one used in the identification of peptide ligands (mimotopes) for antibodies described by Geysen et al. (1986). In principle, a library comprising all possible peptide sequences of a given length made up from the 20 natural amino acids is devided into a full set of pools (sublibraries). Each pool is characterized by one or more defined positions in the context of the randomized other positions. A full set of pools thus covers all combinations of amino acid residues at the defined positions. These pools are synthesized as individual spots on a cellulose paper support (Frank, 1992; Frank et al., 1995) and assayed for activity as an array of immobilized peptide substrates. Our starting array of 400 pools contained two defined positions and had the structure Ac-XXX12XXX, in which the Xs represent mixtures of all 20 natural amino acids and 1 and 2 stand for individual amino acids. The best two amino acids 1 and 2 from the first evalulation were used throughout the second-generation array, where two other positions were screened. This procedure was carried out until all positions had been evalulated, followed by a C- and N-terminal extension of the octameric sequences. The method allows the identification of the major determining amino acids of the substrate recognition motifs and the systematic evaluation of every position. In addition to the investigations with peptides immobilized on paper, the kinetics of the most active sequences were determined in solution.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (6000 Ci/mmol) was from Amersham, Germany. Phosphocellulose paper (P-81) and phosphocellulose (P11) were from Whatman. Kemptide was purchased from Sigma. The semiautomatic robot for the synthesis of the peptides on paper was from ABIMED Anlysentechnik, Langenfeld, Germany. The resins used for preparative peptide synthesis were from Rapp Polymere, Tübingen, Germany. All chemicals were of analytical grade. Organic solvents used during peptide synthesis were of HPLC grade.

Generation of the Peptide Arrays on Paper. The synthesis strategy of the peptide arrays on paper was adopted from the method described previously (Frank, 1992, 1994). Briefly, an APS 222 robotic station was used for semiautomatic assembly of the arrays on Whatman 540 paper that had been esterified uniformly with β-alanine followed by a second β-alanine at the position of the spots. Four hundred twenty-five spots (ϕ 3 mm) were arranged at a distance of 4 mm in a 17 × 25 format (6.7 × 9.8 cm). After each amino acid coupling cycle, the paper was treated with a solution of bromophenol blue in DMF (0.1 mg/mL), which resulted in a blue staining of the spots (Krchnak et al., 1988). All 20 natural amino acids (except for Gly) were used in their L-configuration. Fmoc chemistry was employed, with activation by equimolar amounts of diisopropylcarbodiimide and hydroxybenzotriazole. Side-chain protections were as follows: Asp, Glu, Ser, Tyr, and Thr, *t*-Bu; Asn, Gln, and His, trityl; Arg, Pmc; Lys and Trp, Boc. Cysteine is considered problematic in libraries because of its tendency to oxidize. For this reason some groups omit it from their libraries, and others use it in a protected form throughout their assays. We chose the latter option and used cysteine with acetamidomethyl (Acm) protection, which is not cleaved by TFA but confers good solubility in aqueous buffers. The randomized positions (termed X) were generated by applying 0.1 μL of a 30 mM solution of an equimolar mixture of all 20 amino acids onto the paper, which corresponds to an amount of approximately 70% of the number of amino groups on the paper. The reaction time was 1 h. Spotting was repeated 3 times, after which the reaction was complete as judged by a color change of the spots from blue to yellow or green. As published elsewhere, this procedure assures a sufficiently equal representation of all amino acids at these positions, including the more slowly coupling residues such as valine or isoleucine (Frank, 1995; Kramer, 1993). After completion of the synthesis, the N-termini were acetylated with 2% acetic acid anhydride in DMF. The peptides were deprotected by two successive 1-h treatments with DCM/TFA, 1:1, containing 3% triisobutylsilane and 2% water.

Assaying the Peptide Arrays with PKA and PKG. The paper with the peptide array was moistened with ethanol, washed with 50 mL of incubation buffer A (50 mM MOPS, pH 6.9, 200 mM NaCl, 1 mM Mg-acetate, 0.4 mM EGTA, and 1 mg/mL BSA), and kept overnight in 200 mL of this buffer. The buffer was decanted, and the paper was preincubated at 30 °C with 8 mL of fresh buffer A. One hundred microliters of 10 mM ATP and 100 μCi of [γ-^{32}P]-ATP were added. The reaction was started by addition of PKA catalytic subunit (C_α) or cGMP activated PKG. Final enzyme concentrations were 12.5 and 4 nM for PKA and PKG, respectively. The mixture was incubated for 10 min at 30 °C with slight agitation. The buffer solution was decanted and the paper was washed at least 10 times with 100 mL of 1 M NaCl. One hundred milliliters of an 8 M guanidine hydrochloride solution containing 1% SDS and 0.5% β-mercaptoethanol was added, and the paper was sonicated for 1 h at 40 °C to remove background. The paper was washed several times with water and ethanol and subsequently dried. Radioactivity was determined with the PhosphorImager system (Molecular Dynamics). Quantifications were carried out by integrating uniformly sized circular areas in the center of the spots.

Preparative Peptide Synthesis. The solid-phase synthesis of the peptides was carried out on a Milligen 9050 automatic peptide synthesizer employing Fmoc chemistry with TBTU activation. Side-chain protection of the amino acids was as described for the synthesis on paper. Peptides were cleaved from the resin and deprotected by a 3-h treatment with TFA containing 3% triisobutylsilane and 2% water (10 mL/g of resin). After precipitation with *tert*-butyl methyl ether, the resulting crude peptides were purified by preparative HPLC (RP-8) with water/acetonitrile gradients containing 0.5% TFA and characterized by amino acid analysis and MALDI-MS.

Enzymes. The catalytic subunit (C_α) of PKA was prepared in accordance with a previously described method (Slice & Taylor, 1989; Yonemoto et al., 1991). In brief, the enzyme was expressed in *Escherichia coli* Bl21De3 cells transformed with a pet-3a vector containing the coding sequence of the catalytic subunit. After IPTG induction the cells were incubated at 27 °C for 4−6 h. The bacterial pellet was lysed with a French press, and the enzyme in the soluble fraction was purified over phosphocellulose resin (P11, Whatman) to homogeneity. Peak fractions contained the recombinant catalytic subunit with a typical specific activity of 20 μmol

min^{-1} mg^{-1}. PKG was obtained according to a methods described by Landgraf and Hofmann (1989) and Feil et al. (1993).

Phosphotransferase Activity. Kinase activity was measured for 1.5 min at 30 °C in a final volume of 100 μL containing 20 μL buffer B (250 mM MES, pH 6.9, 2 mM EGTA, 5 mM Mg-acetate, and 50 mM NaCl), 10 μL of BSA (10 mg/mL), 10 μL of DTT (100 mM), 10 μL of [γ-^{32}P]-ATP (1 mM; specific activity, 300−400 cpm/pmol), 10 μL of cGMP (1 mM) or water, 10 μL of appropriate peptide solution, and 20 μL of PKA or PKG (5 mM TES, pH 7.0, 0.2 mM EDTA, and 0.5 mg/mL BSA) to final concentrations of 1 and 2.6 nM, respectively. Fifty-microliter aliquots were spotted on 2 × 2 cm phosphocellulose paper strips (P-81 Whatman), extensively washed in 75 mM phosphoric acid, and the dried strips were subjected to scintillation counting according to Ruth et al. (1991). Peptide concentrations ranged from 100 nM to saturating levels, and all assays were run in duplicate. K_m and V_{max} values were derived from linear regression analysis (Eadie−Hofstee plots).

RESULTS

Development of Conditions for Peptide Phosphorylation on Cellulose Paper. In order to optimize the assay conditions, we first synthesized the array Ac-RAARR1S2 which contained the general substrate motif RRXSX of PKA (Kemp & Pearson, 1990). All 400 combinations of the 20 natural amino acids were used at positions 1 and 2. Initially the commonly used procedure for the investigation of peptide phosphorylation was employed (Hardie, 1993) in which peptides are incubated with the kinases in low-salt buffers followed by an immobilization on phosphocellulose paper and subsequent removal of excess radioactive ATP with 75 mM phosphoric acid. We found that under these conditions ATP bound ionically to sequences on the paper containing basic amino acids and could not be washed off efficiently. However, removal was successful by washing the paper repeatedly in high-salt buffer (1 M NaCl). We also found that the incubation buffer should contain at least 100 mM salt; otherwise, misleading results were obtained because the phosphorylation of peptides that bind ATP ionically was partially inhibited.

Peptide Phosphorylations on Cellulose Paper. After the assay conditions were optimized, an array of the general type Ac-XXX12XXX was used for both kinases, where X represents an equal distribution of all 20 natural amino acids (Cys was used in its Acm-protected form). The whole array represents a library of all 2.56×10^{10} possible octamers, and each sublibrary with defined amino acids 1 and 2 consists of 6.4×10^7 sequences. Incubation with PKA and PKG gave similar phosphorylation patterns with differences between the best dipeptide motifs (Figures 1 and 2A,B). PKA favored the substrates Ac-XXXRRXXX and Ac-XXXRKXXX, whereas PKG was more selective for Ac-XXXRKXXX. The results indicate that two neighboring basic amino acids are the most strongly determinant residues for both kinases.

Based on the results of the first array, the second generation consisted of the structures Ac-XXXRR12X and Ac-XXXRK12X for PKA and PKG, respectively. Both kinases phosphorylated mainly sequences with Ser or Thr at position 2, Ser being generally favored (Figure 2C,D).

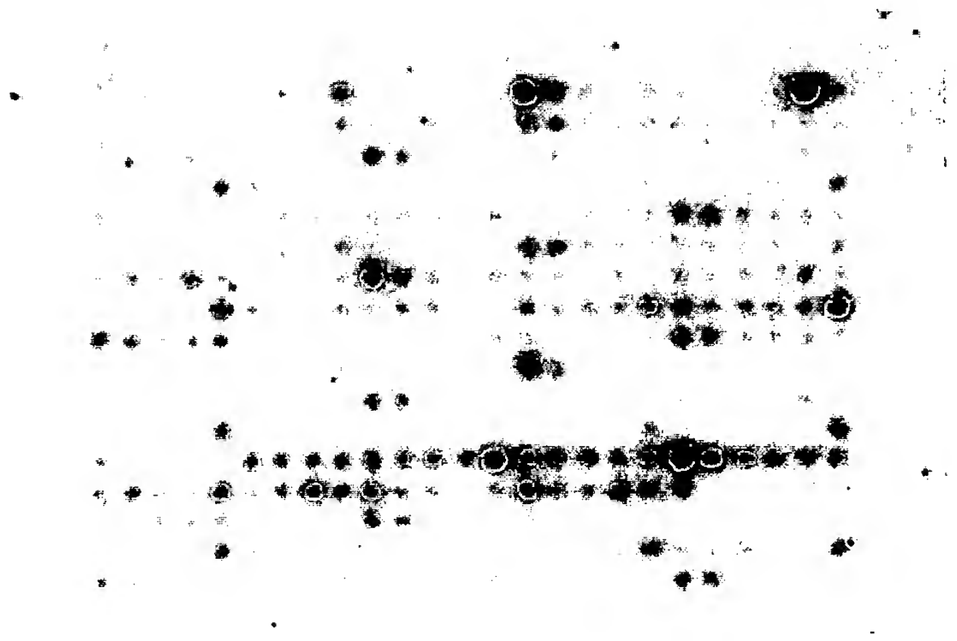

FIGURE 1: PhosphorImager scan of the paper with the array Ac-XXX12XXX after phosphorylation by PKA. Four hundred twenty-five spots are arranged in a 17 × 25 format. Rows 2−17 contain 400 sublibraries in an arrangement where 20 consecutive spots have a particular amino acid at position 1, and position 2 is one of the 20 amino acids, both positions being varied in alphabetical order (according to the single-letter code). For a preliminary estimation of the scattering, the first row contains 25 of the sequences that are used in rows 2−17.

This suggests that position 2 is the site of phosphorylation, which is obvious from a comparison with the general motif for PKA. PKA is less discriminative at position 1, where it shows a slight preference for Ala, whereas PKG clearly prefers Lys. In addition, the arrays Ac-X12RRXXX and Ac-X12RKXXX were used for screening the two positions N-terminal to the basic amino acids. There was no significant preference for any particular amino acid, although PKG had a slight tendency for basic residues (data not shown).

With the third-generation array we screened the structures Ac-X12RRASX and Ac-X12RKKSX for PKA and PKG, respectively (Figure 2E,F). As expected from the second-generation arrays and from previous investigations with soluble peptides (Glass, 1990; Mitchell et al., 1995) neither kinases discriminated strongly at both positions.

Next we used the array Ac-1AERKAS2 for PKA. The best substrate contained Arg at position 1 in combination with Ile at position 2 (Figure 3A), which is again in very good agreement with kinetic observations (Scott et al., 1986). The preference for Arg at position 1 is also consistent with the crystal structure of the PKA/inhibitor peptide complex (Knighton et al., 1991b). Proline at position 2 is particularly disfavored. For PKG we screened the array Ac-1KARKKS2 (Figure 3B). Here, Gln at position 1 and Asn at position 2 were favored. Again, Pro at position 2 was particularly disadvantageous.

In the fifth generation, the array for PKA had the structure Ac-1RAERKASI2 and extended the original octameric pattern by one residue N- and C-terminally. Lys at the N-terminus in combination with Tyr at the C-terminus was the favored combination (Figure 3C). The paper also contained the unextended octameric sequence Ac-RAERKA-SI (10 times) from the previous array. Several of the decapeptides were better substrates than the octapeptide, showing that the affinity has been improved by extending the peptide length. In addition, the peptides Ac-RAERRASI and Ac-RAERRASF were synthesized several times on the paper to reinvestigate the question of whether the middle section of the motif should be Arg-Arg or Arg-Lys and whether Ile or Phe would work best at the C-terminus (Figure 3C, lower part). The tendency from the first-generation array

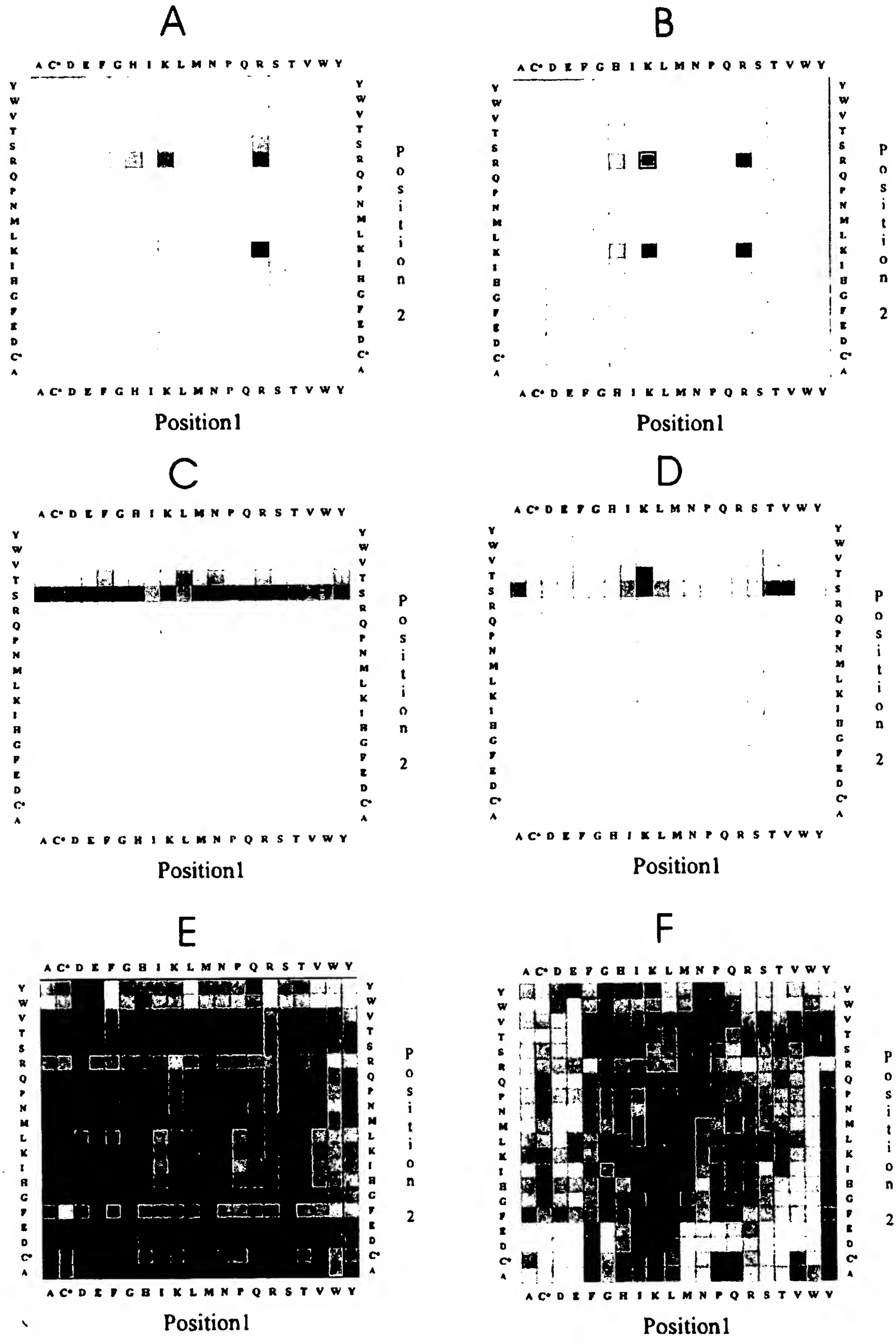

FIGURE 2: Quantified patterns of phosphorylation of the arrays Ac-XXX12XXX (A), Ac-XXXRR12X (C), and Ac-X12RRASX (E) by PKA and of the arrays Ac-XXX12XXX (B), Ac-XXXRK12X (D), and Ac-X12RKKSX (F) by PKG. The shading of each square corresponds in a linear dependence to the amount of phosphorylation of the corresponding spot on the paper.

that the middle motif Arg-Arg is superior to Arg-Lys was confirmed in this statistical comparison. The second conclusion was that the N-terminal Ile is preferred to Phe. The array Ac-1QKARKKSN2 screened with PKG revealed that

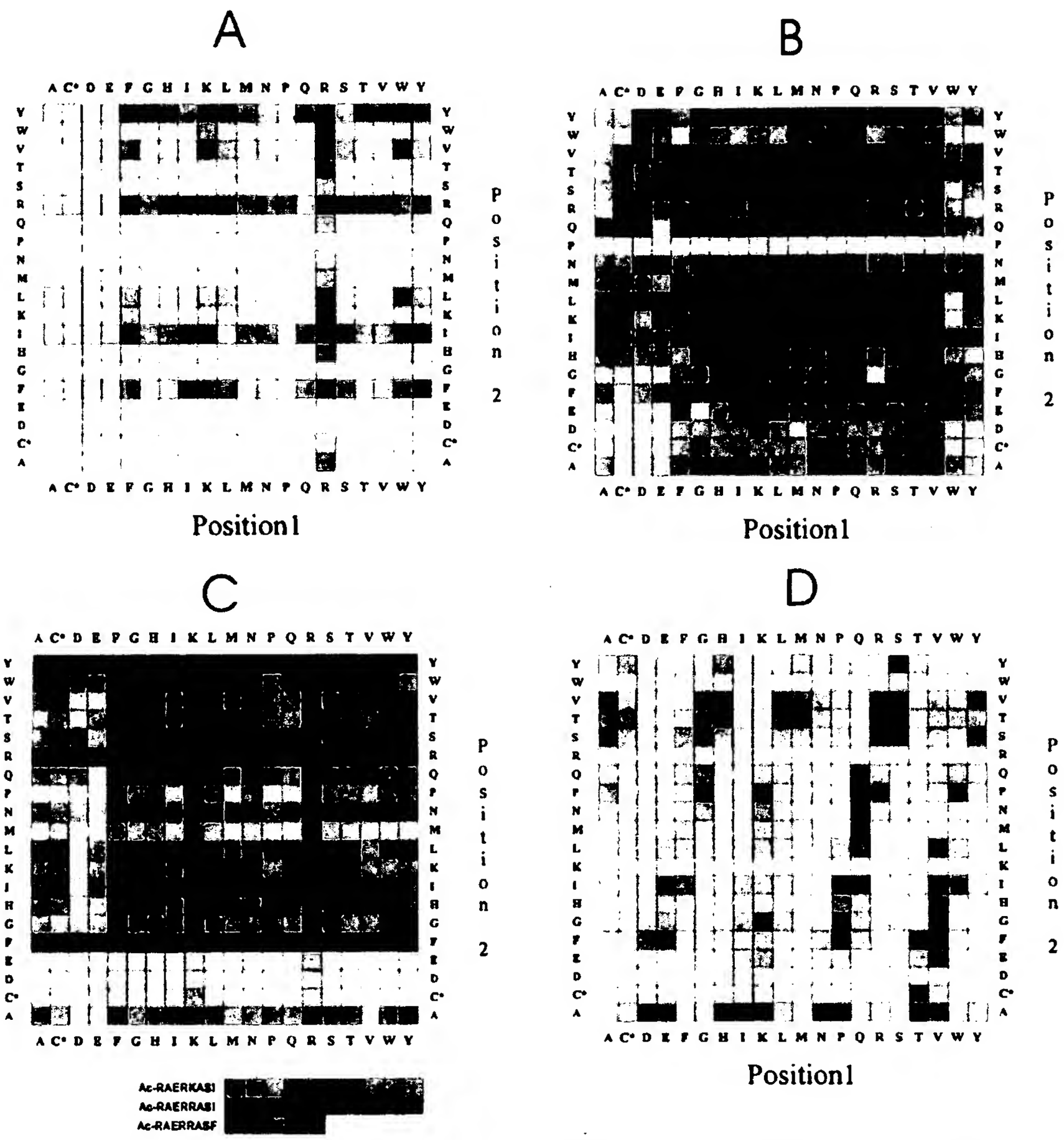

FIGURE 3: Quantified patterns of phosphorylation of the arrays Ac-1AERKAS2 (A) and Ac-1RAERKASI2 (C) by PKA and of the arrays Ac-1KARKKS2 (B) and Ac-1QKARKKSN2 (D) by PKG. The part below array C shows the phosphorylation of the three octameric peptides Ac-RAERKASI, Ac-RAERRASI, and Ac-RAERRASF by PKA.

Thr at position 1 and Ala at position 2 is the best substrate (Figure 3D).

In order to address the question of how reliably the phosphorylation patterns can be interpreted from an array where every sequence or sublibrary is present only once, five of the best octameric sequences from the fourth-generation PKG assay were assembled 10 times each on an extra paper (Figure 4). A scattering of up to 30% around the median value was found. The peptide density of the spots is more uniform (4.5 nmol) with a deviation of up to 10% (right lane in Figure 4). The assay conditions thus obviously add to the scattering. A comparison of short and long phosphorylation times (3 min versus 18 h) gave no differences in the deviation. In this statistical comparison, the sequence with Val at position 1 and Glu at position 2 (sequence 3, Figure 4) was on average better than the combination that was identified initially with the octameric array (sequence 1). A comparison of sequences 1 and 4 in Figure 4 showed that with these particular sequences Arg-Arg in the middle positions worked better than Arg-Lys, which had been found as the best combination on the very first array.

On the basis of the results for both kinases obtained so far, we reexamined the clustering of basic amino acids N-terminal of the phosphorylation site. Forty-three sequences were synthesized 5 or 10 times each as substrates for PKA. The comparison (Figure 5) suggested the following general rules for the specificity of the enzyme: in the general motif P^{-6}-P^{-5}-P^{-4}-P^{-3}-P^{-2}-P^{-1}-S-P^{+1}-P^{+2}, (i) P^{-3} and P^{-2} can be Arg-Arg or Arg-Lys but not Lys-Arg or Lys-Lys (with Lys-Lys being worse than Lys-Arg; Arg-Arg is in most cases better than Arg-Lys); (ii) P^{-1} can be Ala or Lys but not Arg; and (iii) at position P^{-4} Ala and Lys are better than Arg.

A similar investigation with PKG (Figure 6) resulted in the following rules: (i) at position P^{-3} Arg is much better than Lys; (ii) at P^{-2} Lys is in most cases better than Arg; and (iii) at P^{+1} Lys does not effect the activity very much, whereas at P^{+2} Lys is disadvantageous. In most cases AK was superior to KA at positions P^{-4} and P^{-5}. Particularly striking is the high activity of the sequence Ac-TQKARKK-

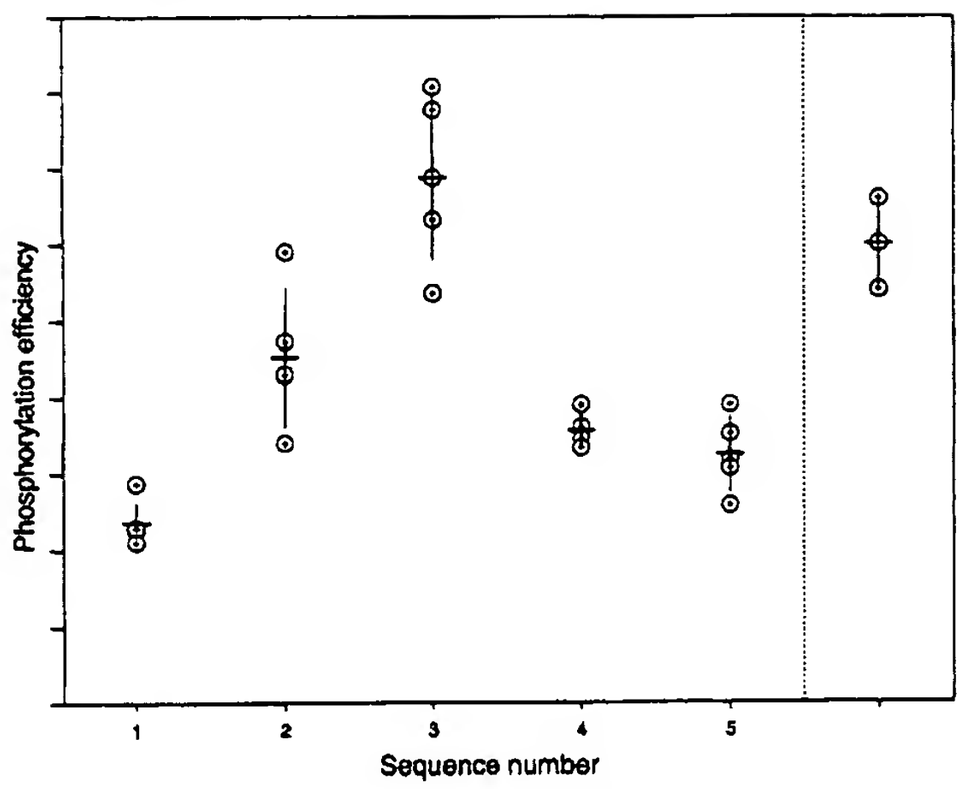

FIGURE 4: Phosphorylation of the following sequences (5 times each) by PKG: 1, Ac-QKARKKSN; 2, Ac-QKARKKSL; 3, Ac-VKARKKSQ; 4, Ac-QKARRKSN; 5, Ac-QKARRKSL. The right lane indicates the scattering of the peptide density between different spots (10%).

SLA (sequence 53, Figure 6). An exchange of Val or Arg at P^{-6} for Gln and of Gln at P^{+1} for Leu had a more dramatic effect than most of the variations of the basic amino acids. This result was unexpected from the systematic sreening of these positions, where both amino acids were inconspicuous. This finding indicates that the specificity at a certain position is influenced by the rest of the sequence.

Kinetic Constants. On the basis of the results from the fourth- and fifth-generation arrays, we synthezised the sequences shown in Table 1 and analyzed them in solution as substrates for the kinases over a wide range of peptide concentrations. Figure 7 shows the initial rate of phosphorylation for peptide 4-3 (Ac-RAERRASI-NH_2) and peptide 5-3 (TQAKRKKSLA-NH_2). Table 1 summarizes the kinetic constants along with kemptide (Whitehouse et al., 1983), *VASP* peptides (Butt et al., 1994), and histone H2B peptide (Glass & Krebs, 1979). Peptide 4-1 (Ac-QKARKKSN-NH_2) was obtained from a screening with PKG and had already proved selective for this enzyme. The K_m was low in comparison to those of peptides known so far and approximately 4-fold lower compared to that of PKA. Peptide 4-2 (Ac-QKARRKSN-NH_2) was used for a comparison of the motifs RKKSX and RRKSX for PKG. Table 1 shows that the substitution in peptide 4-2 does not affect the kinetic constants for PKG very much. Instead it becomes a better PKA substrate, resulting in a loss of specificity. Peptide 4-3 (Ac-RAERRASI-NH_2) was a result from the screening with PKA and confirms basically the well-known consensus sequence (Kemp & Pearson, 1990). From the crystal structure of the catalytic subunit of PKA (Knighton et al., 1991a,b; Zheng et al., 1993) the importance of Arg at position −6 of the peptide inhibitor is known which specifically interacts with residues of the lower lobe of the enzyme and could well explain the lower K_m of peptide 4-3 as compared to kemptide. The SPOT-screening method specifically picked up this Arg as the N-terminal residue. The sequence of peptide 5-3 was a result of the fifth-generation screening on the paper in combination with the statistical comparisons. The changes made between the fourth- and fifth-generation peptides have improved the K_m and the V_{max} by 5-fold (peptide 4-1 versus peptide 5-3). Peptide 5-3 proved to be a remarkably good substrate for PKG in terms of K_m (1.7 μM), catalytic rate (11 μmol/min/mg), and enzyme specificity (6.4). However, the same peptide proved to be a reasonable substrate for PKA as well. This observation was unexpected since the substrate recognition sequence in peptide 5-3 is clearly altered in comparison to peptide 4-3 which contains the well-defined PKA motif.

DISCUSSION

This paper describes the successful application of peptide libraries, displayed as arrays on cellulose paper, for the determination of the amino acids that are the major contributors to the substrate recognition motifs of cAMP- and cGMP-dependent protein kinases. These two kinases have been used as model enzymes for a number of reasons. First, PKA has a well-defined recognition motif. Thus, the literature data could serve as a measure of the performance of our approach and as a valuable guide during the development of the assay conditions. Second, the recognition motif for PKG is less unequivocal, although the two kinases are closely related to each other and share many similar features. Therefore, this approach was used to extend our understanding of the differences in substrate recognition between the two kinases. The recognition motif RRXS of PKA was confirmed by this approach and was used to ensure the reliability of the method. More important, a new substrate sequence (TQAKRKKSLA-NH_2) for PKG with the lowest known K_m value of 1.7 μM and a relatively high V_{max}/K_m ratio of 6.4 was identified. This hitherto unknown PKG substrate recognition motif, KRKKSL, seems to contain sequence parts from known PKG substrate peptides such as the histone H2B peptide RKRSRKE and the protein kinase inhibitor (PKI) peptide GRTGRRNSI (Mitchell et al., 1995). It appears that PKG requires more basic residues compared to PKA, particularly N-terminal of the phosphorylation site as was pointed out by Kennelly and Krebs (1991). Of the known *in vivo* substrates for PKG, histone H2B (chicken, rat) contains the KRKKS motif (Glass, 1990). In addition, the small molecular weight G-protein rap1b (human platelet), which is phosphorylated by PKA and PKG (Siess et al., 1990; Butt & Walter, 1994), contains the recognition sequence KARKKS, which scored extremely high in our screening system (Figure 6). Other *in vivo* and *in vitro* substrates seem to follow variations of the consensus sequence R/K_{2-3}-X/K-S*/T* (22 sites phosphorylated by PKG were examined). The motif KRKKS was also used to screen the SwissProt protein data bank. Several potential new candidates, i.e., prostaglandin receptor type E_3 (mouse) and somatostatin receptor type 2 (human) for *in vivo* phosphorylation by PKG, were identified and are currently being evaluated.

Recently, two other approaches have been decribed for the evaluation of peptides as protein kinase substrates (Wu et al., 1994; Till et al., 1994). Both are based on the generation of peptide libraries on beads by the concept of split synthesis, where each bead carries only one sequence. One study has used penta- and heptapeptide libraries for the determination of the substrate specificity of PKA (Wu et al., 1994). Identification of the beads with the most active substrates was carried out via the incorporation of radioactive phosphate. Since the sequence of the peptide on a particular bead is not known in itself, the peptides had to be analyzed by sequencing. A complete evaluation of all amino acids at the different positions of the library is impossible, and therefore, the approach has to concentrate on the most active

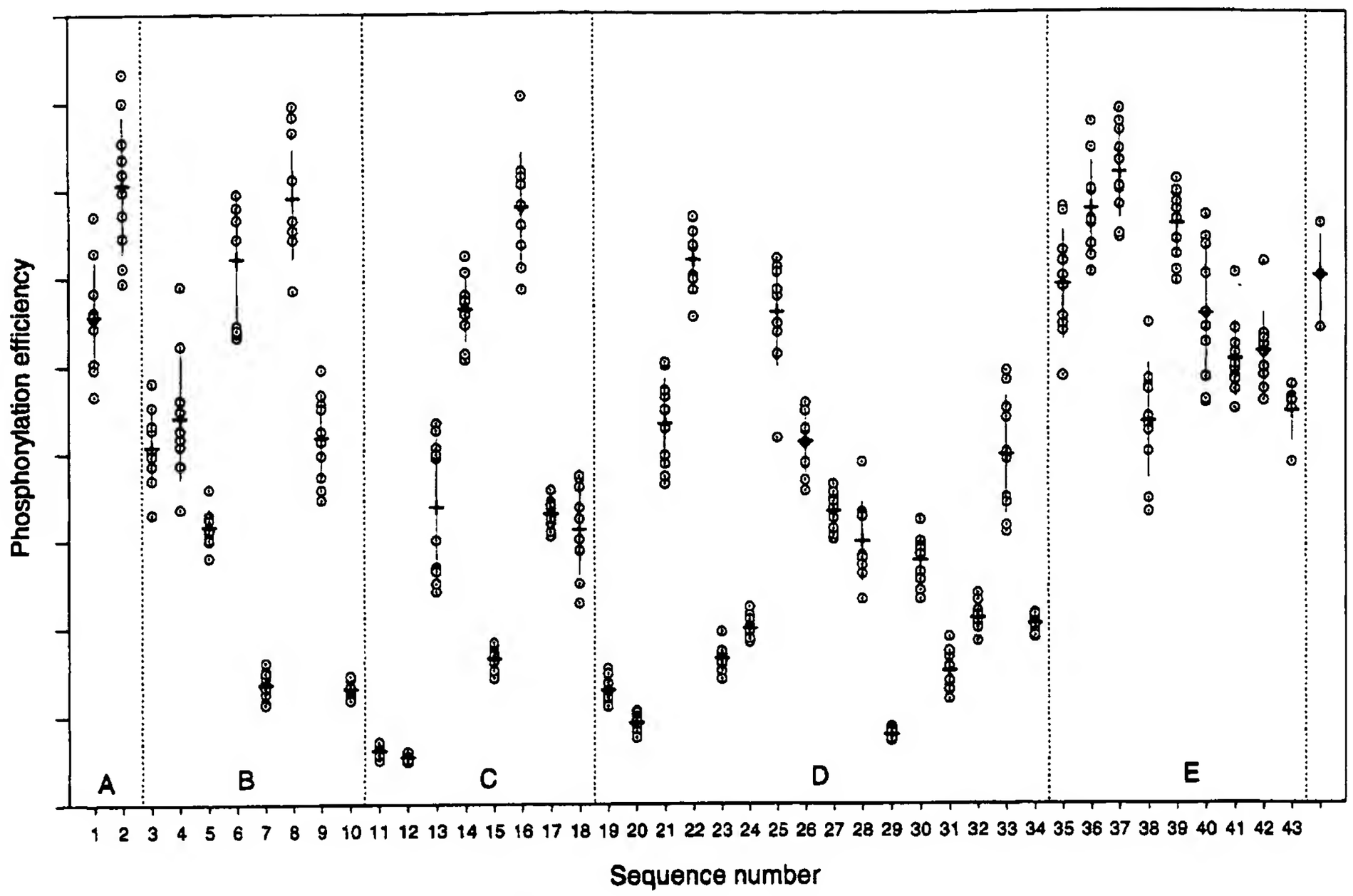

FIGURE 5: Phosphorylation of 43 different sequences (5 or 10 times each) by PKA (all peptides are acetylated at the N-terminus). (A–D) Sequences containing the following structures, where B can be arginine or lysine: A, ABBAS; B, ABBBAS; C, ABBBS; D, ABBBBS. (E) Different variations. Sequences: 1, ARAARKASIY; 2, ARAARRASIY; 3, ARARRRASIY; 4, ARARRKASIY; 5, ARARKRASIY; 6, ARAKRRASIY; 7, ARARKKASIY; 8, ARAKRKASIY; 9, ARAKKRASIY; 10, ARAKKKASIY; 11, ARAAKKKSIY; 12, ARAAKKRSIY; 13, ARAAKRKSIY; 14, ARAARKKSIY; 15, ARAAKRRSIY; 16, ARAARRKSIY; 17, ARAARKRSIY; 18, ARAARRRSIY; 19, ARAKKKKSIY; 20, ARAKKKRSIY; 21, ARAKKRKSIY; 22, ARAKRKKSIY; 23, ARARKKKSIY; 24, ARAKKRRSIY; 25, ARAKRRKSIY; 26, ARARRKKSIY; 27, ARAKRKRSIY; 28, ARARKRKSIY; 29, ARARKKRSIY; 30, ARAKRRRSIY; 31, ARARKRRSIY; 32, ARARRKRSIY; 33, ARARRRKSIY; 34, ARARRRRSIY; 35, KRAKRRASIY; 36, KRAARKASIY; 37, KREKRRASIY; 38, KREKRRASIR; 39, KREKRRASIF; 40, RREKRRASIF; 41, RRDKRRASIF; 42, RRKDRRASIF; 43, RRKERRASIF. The right lane indicates the scattering of the peptide density between different spots (10%).

members in a library. In the second study, derivatives of the heptapeptide kemptide degenerate at only one particular position were investigated as substrates for PKA, and another set of peptides with one degenerate position were used as substrates for the tyrosine kinase v-Abl (Till et al., 1994). Analysis of phosphorylated peptides was carried out by a coupled HPLC–ESMS system and a phosphate-selective stepped collision energy mass spectrometry method. The analysis was carried out with mixtures containing 38 different peptides. An extension to more complex pools is limited because of the degeneracy of the molecular masses if more than one amino acid position is unknown.

Our SPOT approach has the advantage that every amino acid at every position of the sequence can easily be evaluated and compared. This is important if, for example, the identification of the best substrate motif is not desired but rather determining the largest differences between enzyme activities is the major goal. Also, if more than one phosphorylatable amino acid is present in a sequence, each amino acid's individual function as a phosphate acceptor needs to be evaluated. For this purpose individual spots can easily be punched or cut out of the array and analyzed. Peptide quantities per spot are in the range of 5 nmol, which is sufficient for microsequencing or amino acid analysis.

In general, for a successful applicaton of methods based on short linear peptides, the site of phosphorylation (or generally the chemical modification) and at least some of the major contributors of the recognition motif must fit into the length of the peptide that is evaluated. It is possible that additional major contributors of the motifs of both kinases lie outside the range that has been investigated so far. It should also be kept in mind that only linear determinants are being identified. Interactions through secondary or tertiary structures that seem to be important for some classes of enzymes cannot been investigated with short peptides.

Because of the scattering that is inherent in this approach, a statistical comparison of the best sequences that have been identified in a first screening seems necessary in order to determine the best substrate, especially if only small differences among several sublibraries or sequences are present. The specificity at certain positions may be influenced to some extent by the amino acids around that site. This implies that an iterative procedure like the one presented here may not necessarily lead to the absolutely best peptide sequence, but to one that can be expected to contain the major contributors of a motif. It seems generally desirable to increase the number of sublibraries per surface area which would allow the presentation of more defined peptide pools to the enzymes. Simultaneous evaluation of three or more positions would reduce the problem of the sequence dependency of the specificity at a certain position.

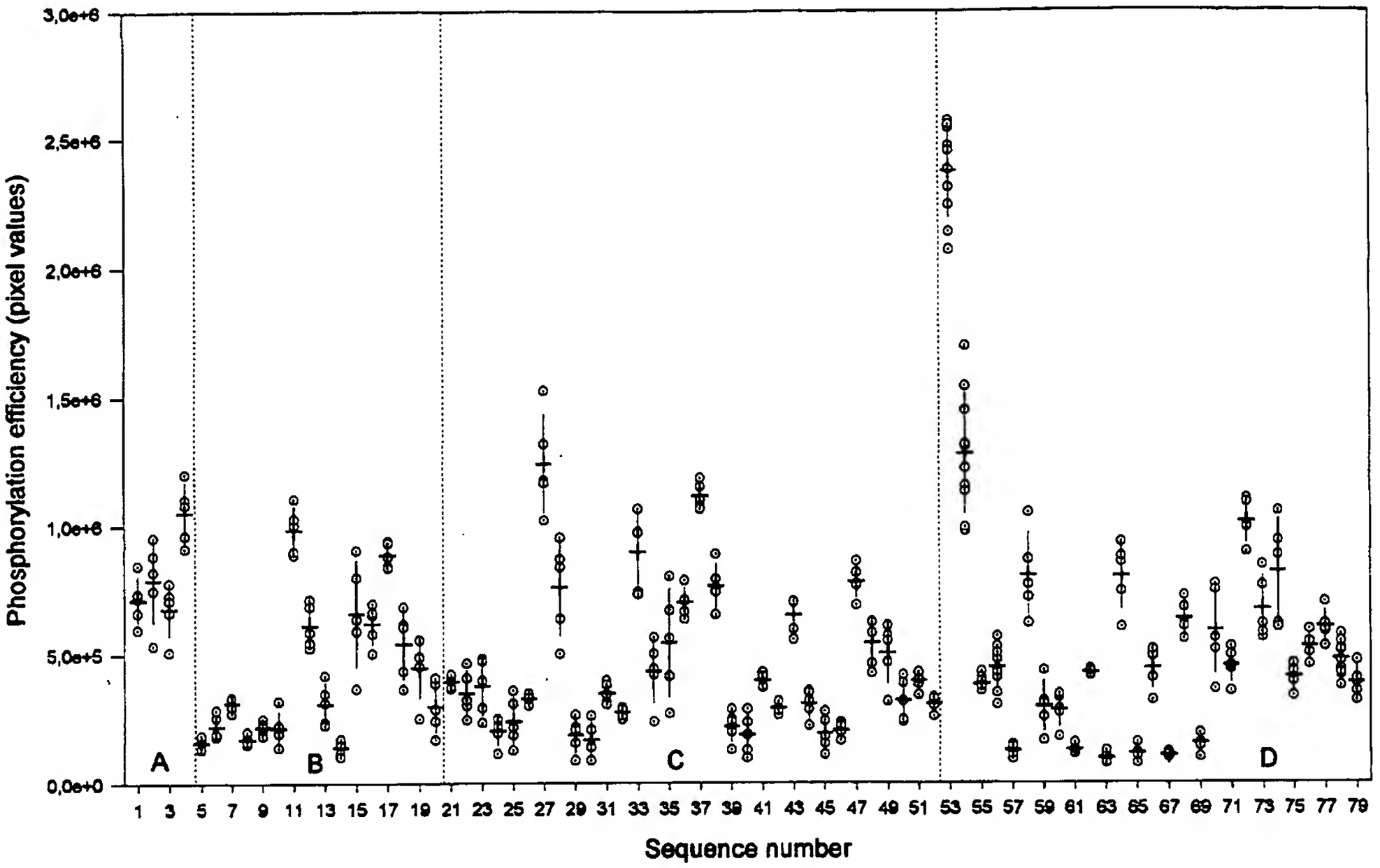

FIGURE 6: Phosphorylation of 79 different sequences (5 or 10 times each) by PKG (all peptides are acetylated at the N-terminus). (A−C) Sequences containing the following structures, where B can be arginine or lysine: A, ABBAS; B, ABBBS; C, ABBBBS. (D) Different variations. Sequences: 1, TVKARKASQA; 2, TRKARKASQA; 3, TVKARRASQA; 4, TRKARRASQA; 5, TVKAKKKSQA; 6, TRKAKKKSQA; 7, TVKAKKRSQA; 8, TRKAKKRSQA; 9, TVKAKRKSQA; 10, TRKAKRKSQA; 11, TVKARKKSQA; 12, TRKARKKSQA; 13, TVKAKRRSQA; 14, TRKAKRRSQA; 15, TVKARRKSQA; 16, TRKARRKSQA; 17, TVKARKRSQA; 18, TRKARKRSQA; 19, TVKARRRSQA; 20, TRKARRRSQA; 21, TVAKKKKSQA; 22, TRAKKKKSQA; 23, TVAKKKRSQA; 24, TRAKKKRSQA; 25, TVAKKRKSQA; 26, TRAKKRKSQA; 27, TVAKRKKSQA; 28, TRAKRKKSQA; 29, TVARKKKSQA; 30, TRARKKKSQA; 31, TVAKKRRSQA; 32, TRAKKRRSQA; 33, TVAKRRKSQA; 34, TRAKRRKSQA; 35, TVARRKKSQA; 36, TRARRKKSQA; 37, TVAKRKRSQA; 38, TRAKRKRSQA; 39, TVARKRKSQA; 40, TRARKRKSQA; 41, TVARKKRSQA; 42, TRARKKRSQA; 43, TRAKRRRSQA; 44, TRAKRRRSQA; 45, TVARKRRSQA; 46, TRARKRRSQA; 47, TVARRKRSQA; 48, TRARRKRSQA; 49, TVARRRKSQA; 50, TRARRRKSQA; 51, TVARRRRSQA; 52, TRARRRRSQA; 53, TQKARKKSLA; 54, TQKARRKSLA; 55, TVMPRKKSQA; 56, TVMPRRKSQA; 57, TVMPRRASQA; 58, TVLIRKKSQA; 59, TVLIRKKSQK; 60, TVLIRRKSQA; 61, TVLIRRKSQR; 62, TVLIRRASQA; 63, TVLIRRASKK; 64, TVYGRKKSQA; 65, TVYGRKKSKR; 66, TVYGRRKSQA; 67, TVYGRRKSRR; 68, TVYGRRASQA; 69, TVYGRRASRK; 70, QVKARKKSQP; 71, QVKARKKSKP; 72, PVKARKKSQI; 73, PVKARKKSKI; 74, RVKARKKSQV; 75, RVKARKKSRV; 76, NVKARKKSQA; 77, NVKARKKSRA; 78, QVKARRKSQP; 79, PVKARRKSQI.

Table 1: Kinetic Constants for the Phosphorylation of Synthetic Peptides and Other Peptide Substrates by PKA and PKG[a]

	PKA			PKG			specificity index	
peptide	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m	K_m (μM)	V_{max} (μmol/min/mg)	V_{max}/K_m	$(V_{max}/K_m$, PKG)/$(V_{max}/K_m$, PKA)	ref
4th generation								
4-1: Ac-QKARKKSN-NH_2	31.7 ± 0.8	2.36 ± 0.03	0.074	9.02 ± 2.32	2.19 ± 0.51	0.242	3.26	this study
4-2: Ac-QKARRKSN-NH_2	15.5 ± 0.8	5.6 ± 0.14	0.36	9.95 ± 2.1	2.44 ± 0.23	0.245	0.68	this study
4-3: Ac-RAERRASI-NH_2	1.99 ± 0.19	8.03 ± 0.24	4.04	13.95 ± 2.15	2.55 ± 0.19	0.183	0.045	this study
5th generation								
5-3: TQAKRKKSLA-NH_2	2.68 ± 0.36	7.92 ± 0.22	2.96	1.74 ± 0.06	11.11 ± 0.17	6.39	2.16	this study
kemptide: LRRASLG	4.28 ± 0.94	9.90 ± 0.64	2.31	120	4.5	0.037	0.016	Whitehouse et al. (1983); this study
H2B: RKRSRKE	113	1.18	0.0104	21.6	4.4	0.204	19.6	Glass and Krebs (1979)
VASP: LRKVSKQE	1395	2.6	0.0018	94	3.7	0.039	21	Butt et al. (1994)
VASP: IERRVSNAG	26	2.7	0.104	30	2.2	0.073	0.71	Butt et al. (1994)

[a] Values are given as means of three or four experiments ± SD.

The SPOT approach can be expected to be generally applicable to the elucidation of protein kinase specificity and to the investigation of other enzymatic transformations. Corresponding work is in progress in our laboratory for further improvements of the technique and applications of the method.

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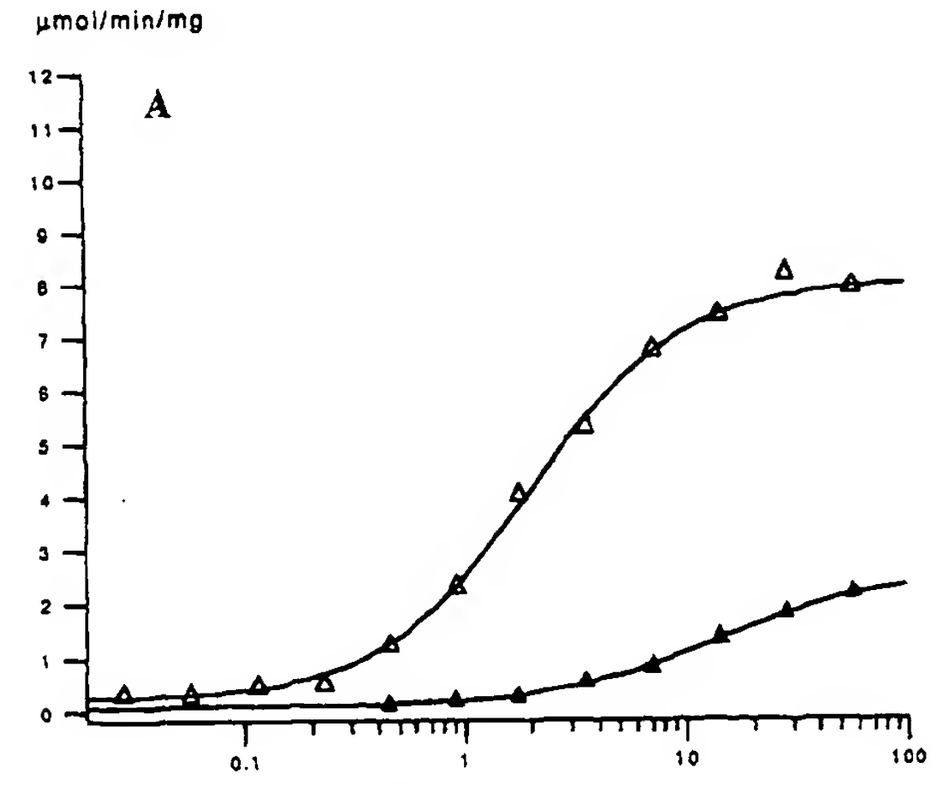

FIGURE 7: Protein kinase activity as a function of substrate concentration. (A) Peptide 4-3 (RAERRASI-NH_2, Table 1) was incubated with PKA (△) and PKG (▲), and (B) peptide 5-3 (TQAKRKKSLA-NH_2, Table 1) was incubated with PKA (▽) and PKG (▼), as described under Experimental Procedures. Initial velocities (µmol/min/mg) were plotted against substrate concentrations.

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